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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE HONORABLE BOARD OF PATENT APPEALS AND
INTERFERENCES


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11/19/99

*In re* the Application of Bach *et al.*
Application No.: 08/986,568
Filed: December 5, 1997
Docket No.: 040388/0110
For: **METHOD FOR TREATING AUTO-IMMUNE DISEASES**

BRIEF ON APPEAL

Appeal from Group 1644

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Washington, DC 20007-5109

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## I. REAL PARTY IN INTEREST

The real party in interest is the assignee of the present application, I.N.S.E.R.M., by virtue of the assignment recorded in the U.S. Patent and Trademark Office on April 3, 1998.



## II. RELATED APPEALS AND INTERFERENCES

Appellant, appellant's legal representative, and the assignee of the present application are not aware of any other appeals or interferences which will directly affect or be directly affected by or have a bearing on the Board's decision in the pending appeal.

## III. STATUS OF CLAIMS

Claims 1, 2, 4-7, 9-13, and 16-18 are pending and are appealed herein. Claims 3, 8, 14 and 15 were canceled. A copy of appellant's pending claims is attached hereto as APPENDIX A.

## IV. STATUS OF AMENDMENTS

No amendments have been filed subsequent to the Final Action.

## V. SUMMARY OF THE INVENTION

Appellants' claimed invention relates to treating spontaneous and ongoing autoimmune disease by administering a therapeutically effective amount of a non-mitogenic, anti-CD3-active compound to a mammal (specification, page 3, lines 26-32). By this treatment one can achieve permanent disease remission, through the induction of antigen-specific tolerance (specification, page 3, last paragraph continuing to page 4, line 5).

## VI. ISSUES ON APPEAL

The issues in this appeal are:

(1) Whether claims 1, 2, 4, 5, 9, 13 and 16-18 are anticipated by Chatenoud *et al.* (1994) under 35 U.S.C. §102(b).

(2) Whether claims 1, 2, 4-7, 9-13 and 16-18 are obvious, under 35 U.S.C. §103(a), over Racadot *et al.*, in view of Gussow *et al.* and Chatenoud *et al.*

## VII. GROUPING OF CLAIMS

The appealed claims stand or fall together.

## VIII. SUMMARY OF THE ARGUMENT

Chatenoud *et al.* (1994) would not have suggested that non-mitogenic anti-CD3 antibody fragments induce a durable state of antigen-specific unresponsiveness. Rather, the Chatenoud article demonstrated that administering anti-CD3 antibody fragments induces transient immunosuppression, while administering the intact anti-CD3 antibody induces a durable state of antigen-specific unresponsiveness. The contemporaneous art also suggested that the durable unresponsiveness, observed by Chatenoud *et al.* (1994), resulted from release of immunoregulatory cytokines. Because administering $F(ab')_2$ fragments does not trigger cytokine release, however, one of ordinary skill in the art would not have expected $F(ab')_2$ fragment therapy, in accordance with appellants' claimed invention, to induce a durable remission of overt autoimmunity. By the same token, Racadot *et al.* did not teach that anti-CD3 therapy can induce a durable remission of overt autoimmunity. Therefore, no combination of references would allow one skilled in the art to formulate the present invention by modifying the monoclonal antibody of Racadot *et al.*

## IX. ARGUMENT

### *A. Chatenoud et al. (1994) would not have suggested that non-mitogenic anti-CD3 antibody fragments induce a durable state of antigen-specific unresponsiveness*

#### 1. The animal-model results reported in Chatenoud et al. (1994) did not link administration of anti-CD3 antibody fragments to anything other than a transient state of non-antigen specific immunosuppression

Chatenoud *et al.* (1994) investigated immuno-intervention of autoimmunity using two non obese diabetic (NOD) mice models. In one model, autoimmune insulin-dependant diabetes

mellitus (IDDM) ensues from progressive loss of self-tolerance to β-cell-associated antigens. In a second model, an accelerated model of IDDM is produced by administering cyclophosphamide (CY) to the mice.

**a) The cyclophosphamide-induced model demonstrated that administration of either anti-CD3 antibody or $F(ab')_2$ fragments of the anti-CD3 antibody induces a transient state of non-antigen specific immunosuppression**

Chatenoud *et al.* (1994) demonstrated that anti-CD3 treatment, started one day before a second CY injection, is highly effective in preventing progression to IDDM. The investigators showed that administration of 5 μg/day for 5 consecutive days produces a 75% reduction in observed diabetes ($p < 0.001$). See Chatenoud *et al.* (1994), Figure 2b.

The investigators also documented that administration of $F(ab')_2$ fragments of the anti-CD3 antibody produces similar immunosuppressive activity, if given at higher doses. A five day regime of 10 μg/day or 50 μg/day produced a 40% and 65% reduction in observed diabetes, respectively ($p > 0.05$ and $p < 0.05$). See Chatenoud *et al.* (1994), Figures 2c and 2d.

These results are consistent with previous data which had revealed that short-term, low-dose anti-CD3 treatment is effective in prolonging the survival of mismatched allografts. See Campos *et al.*, *Transplant Proc.*, 25:798-799 (1993). As with the previous allograft data, the CY-induced diabetes model suggested that short-term, low-dose anti-CD3 treatment is effective for inducing immunosuppression transiently. A determination of the long-term effectiveness of the therapy was precluded because CY treated mice typically die within 50 days of the initial CY administration.

**b) The spontaneous diabetes model demonstrated that administration of the intact anti-CD3 antibody (but not of the antibody fragments) induces a durable state of antigen-specific unresponsiveness**

Chatenoud *et al.* (1994) found that a short-term, low-dose anti-CD3 treatment can restore self-tolerance in adult NOD mice with established diabetes. The investigators showed that administration of 5 μg/day of anti-CD3 for 5 consecutive days to overtly diabetic mice produces a durable remission in diabetes. This remission persisted until the animals were sacrificed at approximately 4 months of therapy (*i.e.*, 8-9 months age).

Also, the observed unresponsiveness was antigen-specific. That is, mice showing anti-CD3-induced remission were not globally immunocompromised, as they retained the capacity to reject skin allografts. In addition, CD3-treated animals did not destroy syngeneic islet grafts, further suggesting the specificity of the anti-CD3-induced remission for the β-cell-associated antigens.

Thus, Chatenoud *et al.* (1994) established that both anti-CD3 antibody and $F(ab')_2$ fragments of the anti-CD3 antibody induce a transient state of non-antigen specific immunosuppression, and that the intact anti-CD3 antibody induces a durable state of antigen-specific unresponsiveness.

The examiner has asserted that the "Chatenoud *et al.* reference teaches the induction of antigen-specific unresponsiveness in NOD mice with full-blown disease . . . by injection of non-mitogenic anti-CD3 monoclonal antibody (mAb) $F(ab')_2$ fragments . . . resulting in complete remission of overt disease." Office Action mailed on December 22, 1998 (hereafter, "December 22 action"), at page 2, lines 18-21. So stating, the examiner overlooks the distinction between the transient state, documented by Chatenoud *et al.* in the context of administering anti-CD3 antibody fragments, and the durable unresponsiveness achieved with appellants' claimed invention.

Moreover, the assertion itself is factually erroneous. As discussed above, Chatenoud *et al.* (1994) examined $F(ab')_2$ fragments in the CY-induced model only. As a matter of fact, the lethality of the CY treatment renders the CY-induced model incapable of illuminating the long-term effectiveness of a therapy. Accordingly, the examiner is wrong to conclude that Chatenoud *et al.* anticipates the appealed claims, and the rejection under §102(b) should be overruled.

According to the examiner, Chatenoud *et al.* (1994) at least suggested that immunotherapy, employing a $F(ab')_2$ fragment of an anti-CD3 antibody, would induce a *durable* remission of overt autoimmunity, as occurs upon administration of intact anti-CD3 antibody in the spontaneous diabetes model. Interpreting the Chatenoud article in this fashion, the examiner is informed by the fact that both anti-CD3 therapy and $F(ab')_2$ fragment therapy achieve a ***transient*** reduction of insulitis in another model, the CY-induced diabetes model.

Thus, the examiner has had to generalize from a similarity of results (transient reduction in insulitis), achieved in one animal model (CY-induced diabetes), to conclude that both therapies also would have been expected to have another effect in common (durable remission of overt autoimmunity), in another model (spontaneous diabetes). Appellants submit, however, that the aforementioned differences between these models would have made this generalization untenable as a matter of fact. As noted, the CY-induced model is incapable of elucidating the long-term effectiveness of a therapy, as CY treated mice usually die within 50 days of CY administration, and the skilled artisan therefore would not have generalized in the manner posited by the examiner.

This generalization also is erroneous as a matter of law, because one skilled in the art, at the time of appellants' invention, would not have expected to obtain a durable, antigen-specific unresponsiveness, upon administration of anti-CD3 antibody fragments. This is supported by the attached declaration under 37 C.F.R. §1.132, by Dr. Terry Strom. Dr. Strom explains that such a durable state, documented contemporaneously by Rapoport *et al.*, *J. Exp. Med.*, 178:87-99 (1993), and Pennline *et al.*, *Clin. Immunol. and Immunopath.*, 71: 169-175 (1994), was attributed to immunoregulatory cytokines. But a systematic release of cytokines is not observed upon administration of anti-CD3 antibody fragments, as these are non-mitogenic.

Thus, one of ordinary skill would have expected the remission induced by treatment with CD3 antibody to be the result of cytokine-mediated immunoregulation. The CD3 antibody used by Chatenoud *et al.*, 145 2C11, is a potent mitogen, *i.e.*, it triggers the release of a variety of cytokines when in injected *in vivo*. Among the cytokines released are TNF, IFN-γ, IL-2, IL-3, IL-4, IL-6, IL-10 and granulocyte-macrophage CSF. See Hirsch *et al.*, *J. Immunol.*, 142:737 (1989); Flamand *et al.*, *J. Immunol.*, 144:2875 (1990); Ferran *et al.*, *Eur. J. Immunol.*, 20:509 (1990); Alegre *et al.*, *Eur. J. Immunol.*, 20:707 (1990); Durez *et al.*, *J. Exp. Med.*, 177:551 (1993); Yoshimoto *et al.*, *J. Exp. Med.*, 179: 1285 (1994). Before the Chatenoud article was published, in 1994, IL-4 was known to prevent the onset of diabetes in NOD mice. See Rapoport *et al. J. Exp. Med.*, 178:87-99 (1993). In addition, three months after the publication of Chatenoud *et al.* (1994), IL-10 also was shown to prevent the onset of

diabetes in NOD mice. See Pennline *et al.*, *Clin. Immunol. and Immunopath.*, 71: 169-175 (1994).

Thus, one skilled in the art would have understood that the durable, antigen-specific unresponsiveness achieved by anti-CD3 therapy resulted from a release of immunoregulatory cytokines, brought on by the mitogenic potential of the full-length antibody. As the examiner has acknowledged, by contrast, Hughes *et al.* showed that $F(ab')_2$ fragments generally are non-mitogenic and, hence, do not illicit cytokine release. By the same token, one skilled in the art would have expected that $F(ab')_2$ fragment therapy according to appellants' claimed invention would not trigger cytokine release and, consequently, would not induce a durable, remission of overt autoimmunity.

Accordingly, it would constitute legal error to base a §103 rejection on the generalization, voiced by the examiner to appellants' counsel, that Chatenoud *et al.* (1994) would have suggested immunotherapy, employing a $F(ab')_2$ fragment of an anti-CD3 antibody, to induce a ***durable*** remission of overt autoimmunity.

*B. Racadot et al. did not teach that anti-CD3 therapy is capable of inducing a durable remission of overt autoimmunity*

According to the examiner, "Racadot *et al.* shows that the same anti-CD3 antibody used by Chatenoud *et al.* to treat diabetes can be used to treat unrelated autoimmune diseases such as multiple sclerosis." December 22 action at page 6, lines 16-18. The examiner acknowledges that "Racadot *et al.* teaches that there were two problems with using the intact murine monoclonal antibody, cytokine release and human antibody (HAMA) generation in the patient." *Id.*, page 6, lines 18-20. The examiner alleges, however, that two other publications suggest how to overcome the limitations described in Racadot *et al.*

Thus, the examiner argues that Chatenoud *et al.* (1994) taught "that the use of $F(ab')_2$ fragments alleviates the problem of cytokine release." *Id.* at page 6, lines 20-21. The examiner further notes that $F(ab')_2$ fragments contain murine framework regions and are capable of inducing a HAMA response in a patient. The examiner suggests that "this problem can be alleviated by humanizing the antibody" as taught by Gussow *et al.* *Id.* at page 6, lines 26-27.

The examiner concludes that "it would have been prima facie obvious to a person of ordinary skill in the art at the time the invention was made to modify the ***effective*** muromonab-CD3 mAb by humanization as taught by Gussow *et al.* in order to alleviate the anti-murine complications taught by Racadot *et al.*" and to further "modify the humanized muromonab-CD3 by pepsin digestion of the entire humanized antibody to generate $F(ab')_2$ fragments to use for treatment as taught by Chatenoud *et al.* in order to eliminate the massive cytokine release associated with treatment using intact anti-CD3 antibodies." December 22 action at page 5, lines 12-18 (emphasis added). The examiner also argues that "[o]ne would have been motivated to combine these reference with a reasonable expectation of success." *Id.* at page 5, lines 18-19.

The examiner errs factually in this regard. First, Racadot *et al.* did not use "the same anti-CD3 antibody used by Chatenoud *et al.*" Racadot *et al.* discussed treatment protocols for patients with multiple sclerosis using muromonab-CD3 (OKT-3). The experiments by Chatenoud *et al.* utilized the hamster IgG, anti-murine CD3 antibody, 145 2C11. Second, Racadot *et al.* did not teach that anti-CD3 therapy is capable of inducing a durable remission of overt autoimmunity. In fact, Racadot *et al.* stated that "[t]he use of muromonab-CD3 in patients with multiple sclerosis appears to be deleterious, with an exacerbation of clinical symptoms in some patients." See Racadot *et al.*, page 204, 1st column. Besides failing to show that muromonab-CD3 therapy induces a remission in multiple sclerosis, Racadot *et al.* did not discuss the durability of the treatment. Because Racadot *et al.* failed to teach the effectiveness of anti-CD3 therapy, no combination of references would allow one skilled in the art to expect that the success of the present invention could be achieved by modifying the monoclonal antibodies of Racadot *et al.*

The examiner's assertion that Racadot *et al.* establishes anti-CD3 treatment as an effective therapy for inducing a durable remission of overt autoimmunity is erroneous.

November 15, 1999
Date

35,087 for
Stephen A. Bent
Reg. No. 29,768

# Appendix I

**WE CLAIM**

1. A method of treating spontaneous and ongoing auto-immune diseases in mammals, comprising administering to a mammal, in need of such a treatment, a therapeutically effective amount of one or more non mitogenic anti-CD3 active compounds to achieve permanent disease remission through the induction of antigen-specific unresponsiveness, i.e. immune tolerance.

2. The method of claim 1, wherein said non mitogenic anti-CD3 active compound is a non mitogenic anti-CD3 antibody.

4. The method of claim 1, wherein said non mitogenic anti-CD3 active compound is a non mitogenic anti-CD3 monoclonal antibody.

5. The method of claim 1, wherein said non mitogenic anti-CD3 active compound is a non mitogenic anti-CD3 monoclonal antibody $F(ab')_2$ fragment.

6. The method of claim 1, wherein said non mitogenic anti-CD3 active compound is highly purified, endotoxin-free.

7. The method of claim 4, wherein said monoclonal antibody is selected from the group consisting of murine or humanized antibody.

9. The method of claim 1, wherein said auto-immune disease is diabetes.

10. The method of claim 1, wherein said auto-immune disease is rheumatoid arthritis.

11. The method of claim 1, wherein said auto-immune disease is multiple psoriasis.

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12. The method of claim 1, wherein said auto-immune disease is multiple sclerosis.

13. The method of claim 1, wherein said active compound is administered by injectable route.

16. The method of claim 1, wherein said non mitogenic anti-CD3 compound is a $F(ab')_2$ fragment.

17. The method of claim 13, wherein said active compound is in the form of an injectable solution delivering between 5 and 20 mg of active compound per day.

18. The method of claim 17, wherein said active compound is in the form of an injectable solution delivering between 5 and 10 mg of active compound per day.

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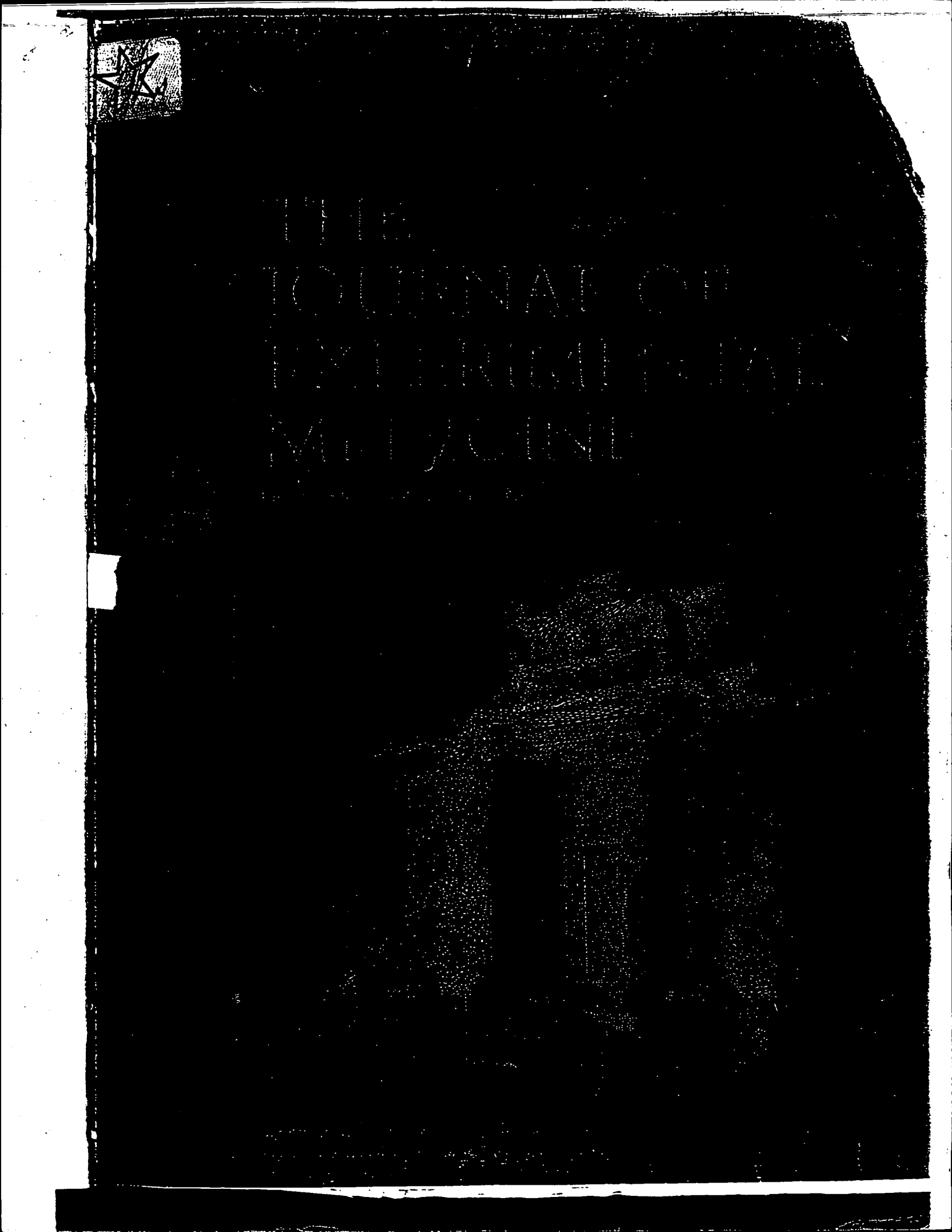

# Interleukin 4 Reverses T Cell Proliferative Unresponsiveness and Prevents the Onset of Diabetes in Nonobese Diabetic Mice

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## Summary

Beginning at the time of insulitis (7 wk of age), $CD4^-$ and $CD8^-$ mature thymocytes from nonobese diabetic (NOD) mice exhibit a proliferative unresponsiveness in vitro after T cell receptor (TCR) crosslinking. This unresponsiveness does not result from either insulitis or thymic involution and is long lasting, i.e., persists until diabetes onset (24 wk of age). We previously proposed that it represents a form of thymic T cell anergy that predisposes to diabetes onset. This hypothesis was tested in the present study by further investigating the mechanism responsible for NOD thymic T cell proliferative unresponsiveness and determining whether reversal of this unresponsiveness protects NOD mice from diabetes. Interleukin 4 (IL-4) secretion by thymocytes from >7-wk-old NOD mice was virtually undetectable after treatment with either anti-TCR $\alpha/\beta$, anti-CD3, or Concanavalin A (Con A) compared with those by thymocytes from age- and sex-matched control BALB/c mice stimulated under identical conditions. NOD thymocytes stimulated by anti-TCR $\alpha/\beta$ or anti-CD3 secreted less IL-2 than did similarly activated BALB/c thymocytes. However, since equivalent levels of IL-2 were secreted by Con A–activated NOD and BALB/c thymocytes, the unresponsiveness of NOD thymic T cells does not appear to be dependent on reduced IL-2 secretion. The surface density and dissociation constant of the high affinity IL-2 receptor of Con A–activated thymocytes from both strains are also similar. The patterns of unresponsiveness and lymphokine secretion seen in anti-TCR/CD3-activated NOD thymic T cells were also observed in activated NOD peripheral spleen T cells. Exogenous recombinant (r)IL-2 only partially reverses NOD thymocyte proliferative unresponsiveness to anti-CD3, and this is mediated by the inability of IL-2 to stimulate a complete IL-4 secretion response. In contrast, exogenous rIL-4 reverses the unresponsiveness of both NOD thymic and peripheral T cells completely, and this is associated with the complete restoration of an IL-2 secretion response. Furthermore, the in vivo administration of rIL-4 to prediabetic NOD mice protects them from diabetes. Thus, the ability of rIL-4 to reverse completely the NOD thymic and peripheral T cell proliferative defect in vitro and protect against diabetes in vivo provides further support for a causal relationship between this T cell proliferative unresponsiveness and susceptibility to diabetes in NOD mice.

During T cell development, the thymus has a central role in the induction and maintenance of immunological self-tolerance, which is a prerequisite for the prevention of autoimmune diseases (1, 2). The mechanisms responsible for this self-tolerance depend on the ability of developing immature thymocytes to proliferative in response to antigenic and/or mitogenic stimuli presented by thymic APCs (1–4). Therefore, the identity and functional status of T cells that are exported from the thymus to the periphery depend on their intrathymic proliferative capacity. Accordingly, if a thymic T cell after it first encounters a self-thymic Ag becomes functionally inactivated and enters a long-lasting state of prolifer-

[1] The first two authors made equivalent contributions to this paper.


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ative unresponsiveness, i.e., a state of anergy, this may lead to changes in the function and/or repertoire of peripheral T cells that could ultimately lead to a breakdown in self-tolerance and autoimmune disease (1–6). Hence, if regulatory T cells that normally confer protection from diabetes become anergic either in the thymus or periphery during the early prediabetic stages of the disease, this event could result in diabetes.

Our previous findings, that thymic and peripheral T cell unresponsiveness after TCR crosslinking correlates with the time of insulitis and persists until the onset of overt disease in nonobese diabetic (NOD)[2] mice (7, 8), raised the possibility that this age-dependent event predisposes to diabetes onset. In this report, we tested this possibility by investigating the mechanisms that elicit thymic and peripheral T cell proliferative unresponsiveness in prediabetic NOD mice and determining whether treatment protocols that reverse this unresponsiveness can protect these mice from diabetes. We analyzed whether $CD4^+$ and $CD8^+$ thymic and splenic T cells from >7-wk-old NOD mice are unresponsive after TCR crosslinking due to a defect(s) in their production of or response to IL-2 and/or IL-4. Interaction between the IL-2- and IL-4-stimulated signaling pathways is important for the regulation of T cell proliferation and maturation, and both IL-2 and IL-4-dependent proliferation of Ag-specific peripheral $CD4^+$ Th0 (produce IL-2 and IL-4) cell clones are sensitive to anergy induction (reviewed in references 8–11). We report that after crosslinking by anti-TCR $\alpha/\beta$ or anti-CD3 mAbs, the secretion of both IL-2 and IL-4 by NOD thymocytes is reduced considerably. Similar results were obtained with anti-TCR/CD3-activated peripheral T cells. However, while the activation of NOD thymic T cells by Con A elicits levels of IL-2 secretion and high affinity IL-2R (HIL-2R) expression/binding capacity comparable to that of control BALB/c mice, their level of IL-4 secretion remains low. By comparison, Con A–activated NOD peripheral T cells secrete significantly less IL-2 and IL-4 than similarly activated BALB/c peripheral T cells. Exogenously added rIL-4 not only restores the in vitro NOD thymic and peripheral T cell proliferative responses to the higher level of control BALB/c thymic and peripheral T cells but also protects NOD mice from developing diabetes in vivo. These observations demonstrate that NOD T cell proliferative unresponsiveness to TCR crosslinking may result from a TCR-mediated defect that leads to decreases in IL-4 and IL-2 secretion. They also raise the intriguing possibility that this thymic and peripheral T cell proliferative unresponsiveness is a causative factor that predisposes to susceptibility to type I diabetes in NOD mice.

## Materials and Methods

*Mice.* In vitro assays of thymic and splenic T cell function were performed using prediabetic male and female inbred NOD/Del mice between 4 and 12 wk of age. These mice were screened for the absence of glycosuria (Diastix; Miles Laboratories, Rexdale, Ontario), and were maintained in the Department of Comparative Medicine's specific pathogen-free mouse facility at the University of Toronto. This colony derived from a breeding nucleus of NOD/LtAlt mice provided by Dr. B. Singh from the University of Alberta (Edmonton, Alberta) breeding colony. The onset of insulitis in NOD/Del mice occurs at 7 wk of age, and diabetes incidence currently is 50–60% in females and 10% in males by 24 wk of age. Age- and sex-matched BALB/cJ mice obtained from the Department of Comparative Medicine mouse facility were used as controls. The specific pathogen-free NOD/Lt and NOD/Jd colonies at The Jackson Laboratory (Bar Harbor, ME) and the Hospital for Sick Children, respectively, were used to study the in vivo effects of IL-4. Diabetes incidence in the NOD/Lt colony is currently 60–80% in females and 40% in males by 24 wk of age, and the incidence in the NOD/Jd colony is currently 92% in females and 10% in males by 24 wk of age.

*Reagents and mAbs.* A cell supernatant containing an mAb (PC61, rat IgG1; reference 12) that detects the HIL-2R was kindly provided by Dr. H. R. MacDonald (Ludwig Institute for Cancer Research, Lausanne, Switzerland). FITC-labeled goat anti-mouse IgG and goat anti-rat IgG (H+L) were purchased from Jackson ImmunoResearch Inc. (West Grove, PA). Ascites containing the H57-597 anti-TCR $\alpha/\beta$ mAb (13) were generously provided by Dr. R. Kubo (National Jewish Center for Immunology and Respiratory Medicine, Denver, CO). The 145-2C11 anti-CD3$\epsilon$ mAb (14) was kindly supplied by Dr. J. Bluestone (University of Chicago, Chicago, IL). The 11B11 anti-IL-4 mAb (15) was kindly provided by Dr. W. E. Paul (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD). The S4B6 anti-IL-2 mAb was kindly provided by Dr. T. R. Mosmann (University of Alberta, Edmonton, Alberta) (16). Murine rIL-2 was kindly provided by Dr. G. Mills (Oncology Research, Toronto General Hospital, Toronto, Ontario). Murine rIL-4 was obtained from either the supernatant of murine IL-4 cDNA–transfected X63Ag8-653 myeloma cells (17) (kindly supplied by Dr. F. Melchers, Basel Institute, Basel, Switzerland), a baculovirus system that expresses a vector containing the murine rIL-4 gene (18) (kindly provided by Dr. W. E. Paul), or from Dr. T. Higgins (Sterling Drug, Inc., Malvern, PA). The IL-4-dependent T cell line CT.4S (19) was also generously supplied by Dr. W. E. Paul. The IL-2-dependent CTLL line (20) was a kind gift from Dr. M. Pierres (Centre d'Immunologie de Marseille-Luminy, Marseille, France). [$^3$H]Thymidine was obtained from Amersham (Oakville, Ontario). [$^{125}$I]rIL-2 (sp act 20–50 $\mu$Ci/$\mu$g) was obtained from NEN (-DuPont Canada Inc., Mississauga, Ontario).

*T Cell Isolation and Activation.* NOD/Del and BALB/c mice were killed by cervical dislocation, thymi and spleens were removed, and single cell suspensions were prepared. Erythrocytes were removed from spleen cell suspensions by treatment for 3 min with 0.1 mM EDTA, 155 mM $NH_4Cl$, and 10 mM $KHCO_3$. Thymocytes and spleen cells were washed and suspended in high-glucose DMEM (HGDMEM) containing 10% heat-inactivated FCS, 10 mM Hepes buffer, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 50 $\mu$M 2-ME (all purchased from Gibco Laboratories, Grand Island, NY). Purified splenic T cells were obtained by passage of the cell suspensions through a nylon wool column. Cells isolated in this way consisted of ≥95% $CD3^+$ T cells, as estimated by fluorescence microscopy using the 145-2C11 anti-CD3 mAb (14). T cells (final concentration, $10^6$/ml) were cultured for 72 h at 37°C in round-bottomed 96-well plates in the presence of 2.5 $\mu$g/ml Con A with or without rIL-2 or rIL-4, and were then used for either flow cytometric anal-

[2] *Abbreviations used in this paper:* HIL-2R, high affinity IL-2 receptor; NOD, nonobese diabetic; SMLR, syngeneic mixed lymphocyte reaction.

yses or assayed for cell proliferation. Alternatively, anti-TCR $\alpha/\beta$– and anti-CD3-induced proliferation were performed by culturing thymocytes for 72 h at 37°C in round-bottomed 96-well plates precoated with a 1:1,000 dilution of ascites containing the H57-597 anti-TCR $\alpha/\beta$ mAb or a 1:200 dilution of hybridoma supernatant containing the anti-CD3 mAb. Irradiated (3,000 rad, $\gamma$ irradiation) and mitomycin C (50 mg/ml; 30 min at 37°C)-treated syngeneic splenocytes (final concentration, $2 \times 10^6$/ml) were added when thymocytes were activated by anti-CD3, as described (7). [$^3$H]Thymidine (1 $\mu$Ci/well) was added 18 h before termination of culture. Cultures were harvested using a 96-well cell harvester (Skatron Inc., Sterling, VA), and the extent of cell proliferation was determined by assay of the amount of [$^3$H]thymidine incorporation using a rack $\beta$ counter (LKB Instruments, Inc., Gaithersburg, MD). IL-2 or IL-4 production by activated thymocytes or splenic T cells ($10^6$ cells/ml) was quantified by culturing cells for 48 h at 37°C in round-bottomed 96-well plates in the presence of either plate-bound anti-TCR $\alpha/\beta$ and anti-CD3 mAbs or Con A (2.5 $\mu$g/ml), and by assay of the culture supernatant for their IL-2 and IL-4 content.

*Flow Cytometric Analysis.* Thymocytes activated by Con A in the presence or absence of IL-2 (800 U/ml) were harvested after 72 h in culture and washed three times in PBS containing 0.01 M $\alpha$-methylmannoside to remove residual Con A. Cells were layered above 30% Percoll and centrifuged at 800 *g* for 20 min to recover viable cells, further washed, and resuspended in PBS containing 0.5% FCS. Viable cells ($2 \times 10^5$) were incubated for 30 min at 4°C in 200 $\mu$l of undiluted anti-HIL-2R mAb–containing supernatant, washed once with PBS plus 0.5% FCS, and further incubated for 30 min at 4°C with 4 mg of FITC-labeled goat anti-rat IgG (H+L) that was previously absorbed on mouse Ig. The stimulated T cell blasts were distinguished from the unactivated cells based on their forward light scatter vs. 90° light scatter characteristics. Stained cells ($10^4$ cells/sample) were enumerated using an Epics V flow cytometer (Coulter Electronics, Hialeah, FL) equipped with a three-decade logarithmic amplifier. Specific staining was obtained after subtracting the background value of second antibody alone from values obtained in the presence of both antibodies.

*IL-2 Binding Assay.* An IL-2 binding assay was performed as described by Robb et al. (21) with minor modifications. Cells were harvested, washed three times, and counted in binding medium (RPMI 1640 supplemented with 5% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 50 $\mu$M 2-ME). They were then resuspended in 3 ml of binding medium, pH 3, for 30 s on ice, washed with ice-cold medium, and resuspended in binding medium. Cells ($0.5–1.0 \times 10^6$) in a final reaction volume of 100 $\mu$l were added in triplicate to flat-bottomed 96-well plates containing twofold serial dilutions of $^{125}$I-labeled human rIL-2 (starting concentration, 4–6 nM). To determine the specificity of binding, unlabeled rIL-2 was added as competitor in 500-fold excess of [$^{125}$I]rIL-2. After incubation for 2 h at 4°C, cell suspensions were transferred to Eppendorf tubes and the bound and free [$^{125}$I]rIL-2 were separated by centrifugation through 100 $\mu$l of an oil mixture (80% dibutyl phthalate and 20% olive oil). The radioactivity in the cell pellets and supernatants containing the bound and the free [$^{125}$I]rIL-2, respectively, was determined in a gamma counter. The number of binding sites per cell and the dissociation constants ($K_d$) of the HIL-2R were calculated by Scatchard analysis.

*IL-2 and IL-4 Secretion Assays.* Secreted IL-2 or IL-4 activity was measured using the IL-2-dependent CTLL line or IL-4-dependent CT.4S cell line, respectively, as described (18, 20). Twofold serial dilutions of test supernatant were added to cultures containing either $1.5 \times 10^4$ CTLL cells or $5 \times 10^3$ CT.4S cells in flat-bottomed 96-well plates in a final volume of 100 $\mu$l/well for 24 h or 48 h, respectively. Cell proliferation was assessed by addition of 1 $\mu$Ci/well of [$^3$H]thymidine 6 h (IL-2) or 18 h (IL-4) before termination of culture, and [$^3$H]thymidine incorporation was determined by liquid scintillation counting.

*IL-4 Treatment In Vivo.* In the first experiment, NOD/Lt females from four separate litters were randomized at 6 wk of age, and a group of 12 females received twice weekly intraperitoneal injections of 500 U (50 ng) murine rIL-4 (sp act, $10^7$ U/mg; Sterling Drug). The control group of 12 females received injections of the vehicle (PBS + 1% serum from 6-wk-old prediabetic NOD/Lt females) to provide carrier protein. The mice were maintained under specific pathogen-free conditions at The Jackson Laboratory and allowed free access to food (Old Guilford 96W pellets; Emory Morse Co., Guilford, CT) and chlorinated drinking water. Mice were tested weekly for glycosuria using Tes-Tape™ (kindly provided by Eli Lilly Co., Indianapolis, IN). Diabetes was diagnosed when mice were glycosuric for at least a consecutive 2 wk. At the end of a 14-wk treatment period, two normoglycemic females from the control and treatment groups, respectively, were killed for analysis, and the remainder of the nondiabetic mice were aged to 52 wk without further treatment. At death, pancreas, submandibular salivary glands, and kidneys from each mouse were fixed in Bouin's solution, embedded in paraffin, sectioned, and stained. Aldehyde fuchsin staining of pancreas sections sampled at three different nonoverlapping levels was used to compare the extent to which insulitic infiltrates had reduced the mass of granulated $\beta$ cells. Splenic leukocyte populations were enumerated by FACScan® (Becton Dickinson & Co., Mountain View, CA) analyses using the anti-Thy-1.2 (clone HO-13-4.9), anti-CD4 (clone GK 1.5), and anti-CD8 (clone 53-6.72) mAbs in ascites form at 1:100, 1:200, and 1:200 dilutions, respectively, as described (7). Analyses of *Escherichia coli* LPS–stimulated IL-1 secretion from peritoneal macrophages and of T cell immunoregulation after activation in a syngeneic mixed lymphocyte reaction (SMLR) were performed as described (22).

In a second experiment, a group of eight 3-wk-old female NOD/Jd mice from two separate litters received twice weekly intraperitoneal injections of 500 U (50 ng in 250 $\mu$l) of a murine rIL-4 (sp act, $\sim 10^7$ U/mg)-containing supernatant derived from Sf9 Drosophila cells infected with a baculovirus that expresses a vector containing the murine rIL-4 gene (18). The control group of six age-matched NOD females received injections of supernatant (250 $\mu$l) derived from Sf9 cells infected with the wild-type baculovirus vector that does not contain the murine IL-4 gene (18). Treatment of both groups of mice was continued for 12 wk until the mice were 15 wk of age, after which treatment ceased. The mice were maintained at the Hospital for Sick Children, tested weekly for glycosuria, and diagnosed for diabetes, as described in the first experiment.

## Results

*Expression of HIL-2R on NOD Con A–activated Thymic T Cell Blasts Is Normal.* Engagement of the TCR/CD3 complex by a mitogenic lectin or Ag/MHC generally leads to a rapid increase in HIL-2R expression followed by IL-2 production and stimulation of T cell proliferation (23). Alternatively, if such TCR stimulation results in a decrease in or lack of expression of HIL-2R, then T cell proliferative unresponsiveness ensues (24). In Con A-activated CD4$^+$ and CD8$^+$

spleen T cells from 10-wk-old NOD/ShiKbe mice, HIL-2R expression is reduced about threefold (25). The latter result raised the possibility that the proliferative unresponsiveness of NOD thymocytes from >7-wk-old mice to Con A stimulation (7) is mediated by a diminished expression of HIL-2R. To test this possibility, NOD and BALB/c thymocytes were cultured in the presence of 2.5 μg/ml Con A for 72 h, and were then analyzed by flow cytometry for their surface expression of HIL-2R. The percentages of these thymocytes that express HIL-2R were very similar (Table 1), and their levels of surface expression of HIL-2R were equivalent (our unpublished data).

Binding of IL-2 to the HIL-2R generally results in an increased level of expression of the HIL-2R (23). To determine whether the post-HIL-2R part of the IL-2 pathway that enhances HIL-2R membrane expression is intact, the level of HIL-2R surface expression in NOD and BALB/c thymocytes stimulated by Con A plus 800 U/ml rIL-2 was assayed. The latter saturating concentration of rIL-2 was chosen since NOD thymocytes proliferate rather poorly in response to lower concentrations of rIL-2 (7). The addition of 800 U/ml rIL-2 increased both the proportions of T cells bearing HIL-2R (Table 1) and the surface densities of these receptors on NOD and BALB/c thymocytes (our unpublished data).

Results obtained by Scatchard analysis of [$^{125}$I]rIL-2 binding to thymocytes supported our estimates of the relative levels of expression of HIL-2R on these cells observed by flow cytometry. The number per cell and affinity of HIL-2R were very similar in both young (<7 wk) and old (>7 wk) Con A-activated NOD and BALB/c thymocytes (Table 2). An age-dependent effect was noted since a twofold decrease in the number of HIL-2R molecules per cell on old vs. young activated thymocytes was observed. No significant difference was observed between the affinity and number per cell of HIL-2R on NOD and BALB/c quiescent thymocytes (data not shown). Thus, the surface density and binding capacity of HIL-2R on activated NOD thymic T cells appears to be normal (i.e., equivalent to that of activated BALB/c thymocytes), and presumably is not responsible for the Con A-mediated proliferative defect of >7-wk-old NOD thymocytes.

*NOD Thymic T Cells Secrete Reduced Amounts of IL-2 after Stimulation by Anti-TCR/CD3 but Not Con A.* Since the level of HIL-2R expression on Con A-activated NOD thymocytes is normal, we analyzed whether the unresponsiveness of these T cells results from a defect in IL-2 production after stimulation through the TCR, as has been observed in Th1 clones (3, 4, 9, 26, 27). The capacity of NOD thymocytes to secrete IL-2 after activation by either anti-TCR $\alpha/\beta$, anti-CD3, or Con A was compared. Anti-TCR $\alpha/\beta$ (Fig. 1 *A*) and anti-CD3 (Fig. 1 *B*) each stimulated ~10-fold less IL-2 secretion by thymocytes from 8–12-wk-old NOD mice than from age- and sex-matched control BALB/c thymocytes. However, the amounts of IL-2 secreted by NOD and BALB/c Con A-activated thymic T cells were virtually identical (Fig. 1 *C*). Thus, mitogen stimulation of NOD thymocytes overcomes the defect in IL-2 secretion that is observed after crosslinking the TCR with an anti-TCR mAb.

*NOD Spleen T Cells Secrete Reduced Amounts of IL-2 after Stimulation by Either Anti-TCR/CD3 or Con A.* To determine whether the reduced IL-2 secretion observed in stimulated NOD thymic T cells is also manifested in activated NOD peripheral T cells, we compared the levels of IL-2 secretion by activated spleen T cells from NOD and BALB/c mice. The level of IL-2 secreted by activated NOD splenic T cells is significantly lower than that noted for activated thymic T cells. This was observed not only for after T cell stimulation by anti-TCR $\alpha/\beta$ (Fig. 1 *D*) and anti-CD3 (Fig. 1 *E*) but also by Con A (Fig. 1 *F*). Thus, while Con A normalizes the defect in IL-2 secretion by NOD thymic T cells, this is not the case for Con A-activated NOD spleen T cells.

*NOD Thymic and Splenic T Cell Proliferative Unresponsiveness Is Associated with Diminished IL-4 Secretion and Is Reversed by Exogenous rIL-4.* Interaction between the IL-2- and IL-4-stimulated signaling pathways plays an important role in

**Table 1.** *Frequency of HIL-2R-bearing NOD and BALB/c Con A–activated T Cell Blasts Is Equivalent*

| Strain | Stimulus | |
|---|---|---|
| | Con A | Con A + IL-2 |
| | % | |
| NOD | 25 | 56 |
| BALB/c | 18 | 49 |

Thymocytes from 8–12-wk-old NOD or control BALB/c mice were incubated for 72 h at 37°C with Con A (2.5 μg/ml) in the absence or presence of rIL-2 (800 U/ml). The recovered viable cells were stained with an anti-HIL-2R mAb and FITC-labeled goat anti-rat Ig (H + L), and the large thymocyte blasts were gated and analyzed. The mean percentages of positively stained thymocytes obtained in three independent experiments are indicated, and the SD of the means were ≤10%.

**Table 2.** *Expression of HIL-2R by Con A–activated NOD Thymocytes Is Normal*

| Strain | Age | Affinity $K_d$ | Molecules/cell |
|---|---|---|---|
| | | *pM* | |
| NOD | Young | 38 | 2,025 |
| BALB/c | Young | 67 | 2,145 |
| NOD | Old | 52 | 1,015 |
| BALB/c | Old | 57 | 1,215 |

Assays of binding of [$^{125}$I]rIL-2 to Con A–activated NOD thymocytes were performed as described in Materials and Methods. Young mice were 4–6 wk of age and old mice were 8–12 wk of age. The number of binding sites per cell and the dissociation constants ($K_d$) of the HIL-2R were calculated by Scatchard analysis. Each result was obtained using a group of two to five mice in three independent experiments.

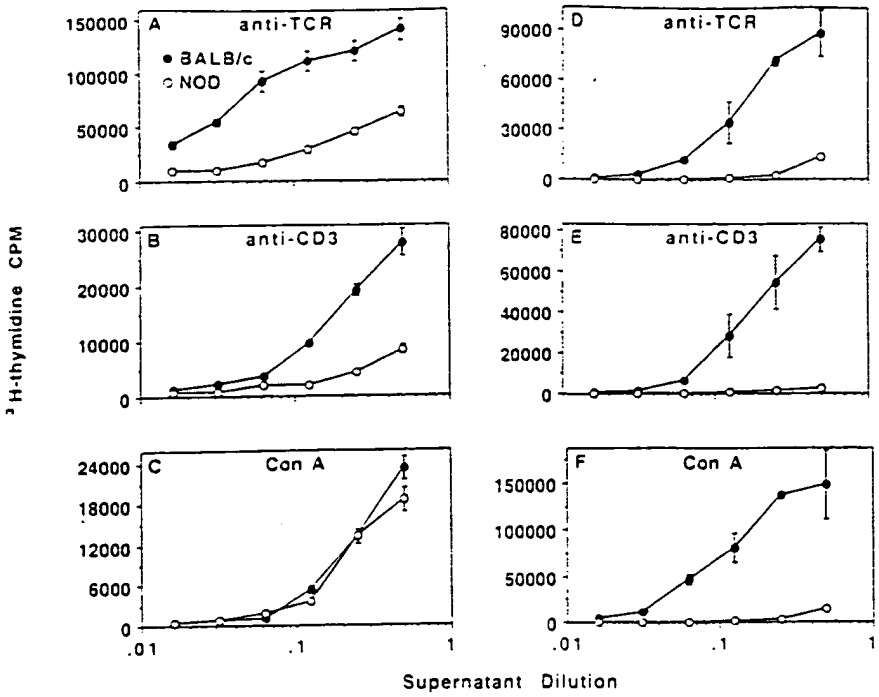


**Figure 1.** IL-2 secretion by activated thymic and splenic T cells. Thymic (*A–C*) and splenic (*D–F*) T cells from 8–12-wk-old NOD and BALB/c mice were activated for 48 h at 37°C by either plate-bound anti-TCR $\alpha/\beta$ (*A* and *D*) and anti-CD3 (*B* and *E*) mAbs, or by 2.5 $\mu$g/ml Con A (*C* and *F*). Culture supernatants were removed and assayed for their IL-2 activity by stimulation of proliferation of CTLL IL-2-dependent T cells. The results of triplicate cultures are expressed as the mean values ± SD, and are representative of three different experiments.

the regulation of T cell proliferation and maturation (28–30). In addition, Th1 and Th2 cells differ in their function and production of autocrine growth factors; IL-2 is produced by Th1 and IL-4 is produced by Th2, respectively (31). We therefore examined the level of secretion of IL-4 by activated NOD thymic and splenic T cells. NOD thymocytes activated by either anti-TCR $\alpha/\beta$ (Fig. 2 *A*) or anti-CD3 (Fig. 2 *B*) did not secrete detectable amounts of IL-4 in comparison with similarly activated thymocytes from age- and sex-matched BALB/c control mice. Con A–activated NOD thymocytes secreted considerably less IL-4 than did similarly activated BALB/c thymocytes (Fig. 2 *C*). Similar results were obtained

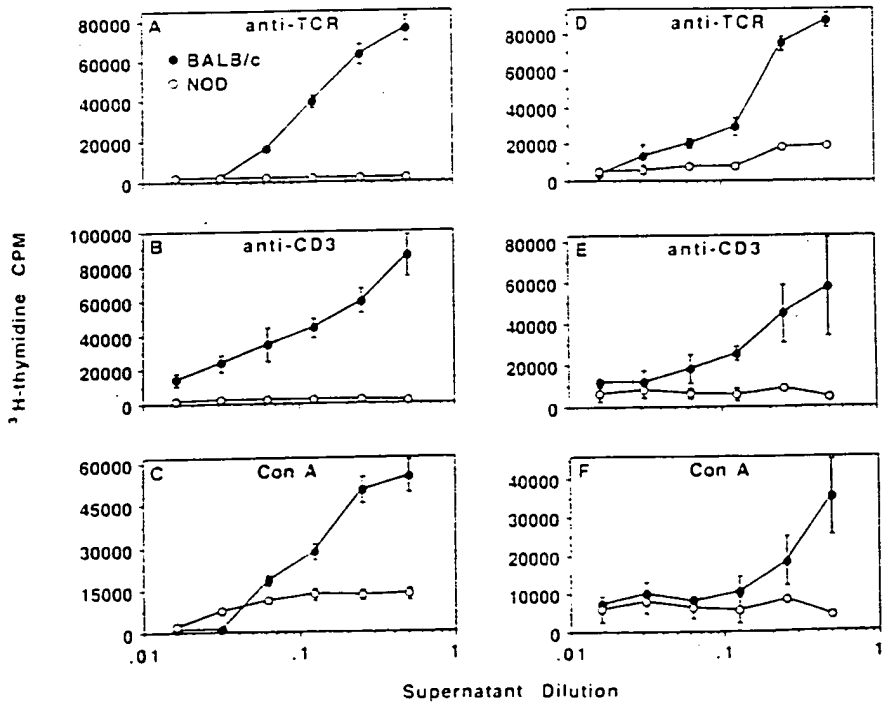


**Figure 2.** IL-4 secretion by activated thymic and splenic T cells. Thymic (*A–C*) and splenic T cells (*D–F*) from 8–12-wk-old NOD and BALB/c mice were activated for 48 h at 37°C by either plate-bound anti-TCR $\alpha/\beta$ (*A* and *D*) or anti-CD3 (*B* and *E*) mAbs, or by 2.5 $\mu$g/ml Con A (*C* and *F*). Culture supernatants were removed and assayed for their IL-4 activity by stimulation of proliferation of CT.4S IL-4-dependent T cells. The results of triplicate cultures are expressed as the mean values ± SD, and are representative of three different experiments.

upon activation of NOD splenic T cells activated by either anti-TCR $\alpha/\beta$ (Fig. 2 *D*), anti-CD3 (Fig. 2 *E*), or Con A (Fig. 2 *F*). Therefore, NOD thymic and peripheral T cells both display a relative inability to secrete IL-4 after activation through either the TCR or by mitogen.

To establish whether this defect in IL-4 secretion is causally related to T cell hyporesponsiveness, the ability of rIL-4 plus either anti-TCR $\alpha/\beta$, anti-CD3, or Con A to stimulate NOD T cell proliferation was assayed. Addition of exogenous rIL-4 restored to normal the proliferative capacity of NOD thymocytes stimulated by anti-TCR $\alpha/\beta$ (Fig. 3 *A*), anti-CD3 (Fig. 3 *B*), or Con A (Fig. 3 *C*). Similarly, exogenous rIL-4 also restored the proliferative response of anti-TCR $\alpha/\beta$–activated NOD spleen T cells to that of activated control BALB/c spleen T cells (Fig. 3 *D*). In contrast, supraphysiological amounts of rIL-2 only partially correct the thymic T cell unresponsiveness to anti-CD3 (Fig. 4) or Con A (7). Therefore, NOD thymic and peripheral T cell proliferative unresponsiveness may be due to diminished IL-4 secretion as a consequence of a defect in the Th2 T cell subset.

*Exogenous rIL-4 Completely Restores Normal IL-2 Secretion by NOD Thymic T Cells, whereas Exogenous rIL-2 Only Partially Restores IL-4 Secretion by NOD Thymic T Cells.* To test whether an increased level of IL-2 secretion is associated with the capacity of exogenous rIL-4 to restore the proliferative responsiveness of NOD T cells, the ability of anti-TCR $\alpha/\beta$ plus exogenously added rIL-4 to stimulate IL-2 secretion by BALB/c and NOD thymocytes was assayed. The amount of rIL-4 added, i.e., 125 U/ml, promotes essentially a maximal proliferative response of anti-TCR $\alpha/\beta$–stimulated NOD thymic T cells (Fig. 3 *A*). Equivalent levels of IL-2 secretion by anti-TCR $\alpha/\beta$ + rIL-4–activated thymocytes from both strains were noted (Fig. 5 *A*). Since the IL-2-dependent CTLL cell line used here may be partially responsive to IL-4, we verified whether CTLL proliferation observed in Fig. 5 *A* was at all due to the presence of residual exogenous rIL-4 present in the supernatants of the activated T cells. The ability of the 11B11 anti-IL-4 mAb to block CTLL and CT.4S cell proliferation was compared. This mAb (10 $\mu$g/ml) reduced the CTLL proliferative response to BALB/c- and NOD-activated thymic T cell culture supernatants by only 33 and 26%, respectively (Fig. 5 *B*). In contrast, this mAb inhibited virtually 100% of the IL-4-dependent CT.4S proliferative response, demonstrating that the CTLL proliferative response obtained in Fig. 5 *A* was stimulated predominantly by IL-2. Thus, the ability of IL-4 to restore the proliferative responsiveness of NOD T cells activated via the TCR is associated with its capacity to restore to normal (i.e., to the BALB/c T cell level) the level of IL-2 secretion by these cells.

To test whether deficient IL-4 secretion mediates the partial restoration of the NOD thymic T cell proliferative response by exogenous rIL-2, the ability of anti-TCR $\alpha/\beta$ plus exogenously added rIL-2 to stimulate IL-4 secretion by BALB/c and NOD thymocytes was assayed. The amount of rIL-2 added, i.e., 250 U/ml, promotes essentially a maximal proliferative response of anti-TCR $\alpha/\beta$–stimulated NOD thymic T cells (Fig. 4). NOD thymocytes secreted considerably less IL-4 than did BALB/c thymocytes after stimulation with anti-TCR $\alpha/\beta$ + rIL-2 (Fig. 5 *C*). Since the IL-4-dependent CT.4S cell line is partially responsive to IL-2 at a concentration of $\geq$50 U/ml (19), we verified whether any CT.4S proliferation observed in Fig. 5 *C* was stimulated by residual exogenous rIL-2 present in the supernatants of the activated T cells. The ability of the S4B6 anti-IL-2 mAb to block CT.4S and CTLL cell proliferation was compared. This mAb (25% of hybridoma culture supernatant) reduced the CT.4S proliferative response to BALB/c- and NOD-activated thymic T cell culture supernatants by only 27 and 33%, respectively. In contrast, this mAb inhibited the IL-2-dependent CTLL proliferative response to BALB/c- and NOD-activated thymic T cell culture supernatants by 86 and 85%, respectively (Fig. 5 *D*). Thus, the inability of IL-2 to completely restore the proliferative responsiveness of TCR-activated NOD thymic T cells is associated with the partial restoration of IL-4 secretion by these cells.

*NOD Thymic T Cells Require a Continuous IL-4 or IL-2 Stimulus to Secrete Normal Levels of IL-2 or Partial Levels of IL-4, Respectively.* Our data described above raise the possi-

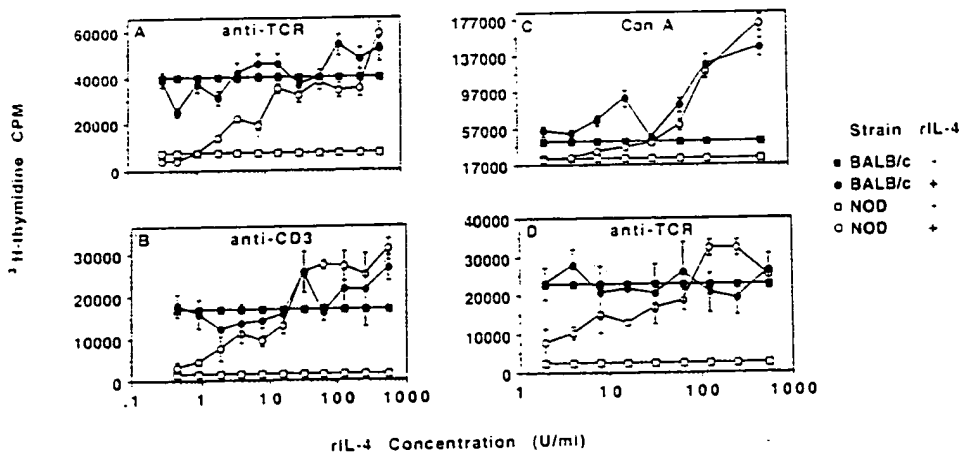


**Figure 3.** Exogenous rIL-4 corrects the proliferative unresponsiveness of activated NOD thymic and splenic T cells. Thymocytes from 8–12-wk-old NOD and BALB/c mice were activated by either plate-bound anti-TCR $\alpha/\beta$ (*A*) and anti-CD3 (*B*) mAbs or by Con A (*C*) in the presence of varying amounts of rIL-4. Splenic T cells were activated by plate-bound anti-TCR $\alpha/\beta$ mAb in the presence of varying amounts of rIL-4 (*D*). Cell proliferation was determined by [$^3$H]thymidine incorporation. The results of triplicate cultures are expressed as the mean values ± SD, and are representative of three different experiments.

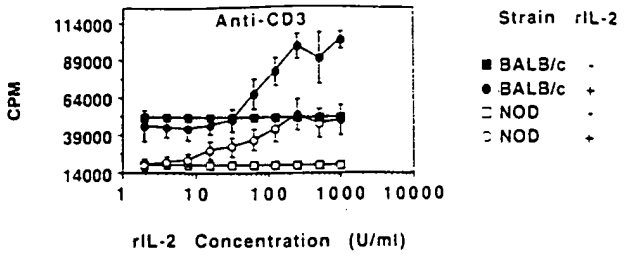


**Figure 4.** Exogenous rIL-2 partially corrects the proliferative unresponsiveness of activated NOD thymic T cells. Thymocytes from 8–12-wk-old NOD and BALB/c mice were activated by anti-CD3 mAb in the presence of varying amounts of rIL-2. Cell proliferation was determined by [$^3$H]thymidine incorporation. The results of triplicate cultures are expressed as the mean values ± SD, and are representative of three different experiments.

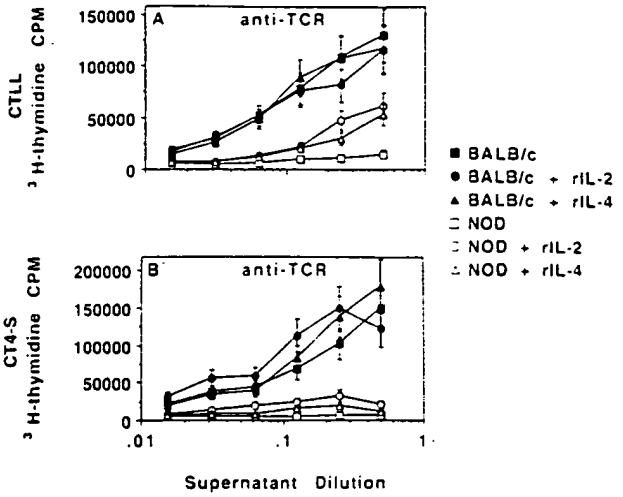


**Figure 6.** NOD thymic T cells require a continuous IL-4 or IL-2 stimulus to secrete normal levels of IL-2 or partial levels of IL-4, respectively. Thymic T cells from 8–12-wk-old NOD and BALB/c mice were activated by anti-TCR $\alpha/\beta$ in the presence of rIL-2 (250 U/ml) or rIL-4 (125 U/ml). After 24 h of culture, cells were harvested, layered above 30% Percoll, and centrifuged at 800 *g* for 20 min to remove dead cells. After washing, viable T cell blasts were incubated for an additional 48 h in 96-well plates coated with anti-TCR. (*A*) Culture supernatants were harvested and assayed for their IL-2 activity. (*B*) Culture supernatants were harvested and assayed for their IL-4 activity. The results of triplicate cultures are expressed as the mean ± SD, and are representative of three different experiments.

bility that NOD T cell proliferative unresponsiveness may be due primarily to a defect in the Th2 T cell subset. Interestingly, Ben-Sasson et al. (18) previously reported that Th2 cells may actually consist of two distinct subsets: one that requires IL-2 for IL-4 production and a second that can produce IL-4 without the requirement of IL-2. Based on this report, we considered that the following two possibilities may explain our findings. First, only the IL-2-responsive NOD Th2 subset produces IL-4 upon stimulation with anti-TCR + rIL-2, and this would account for the partial restoration of endogenous IL-4 production. Second, this IL-2-responsive Th2 subset does not produce sufficient amounts of endogenous IL-4 upon stimulation with anti-TCR + rIL-2, and therefore IL-4 may not be available in the amounts required for complete restoration of the response to be achieved.

To further examine why rIL-2 only partially restores the level of IL-4 secretion and proliferative responsiveness of NOD thymic T cells, we investigated the autocrine effect of rIL-4 on these responses of NOD Th2 cells and compared it with the effect of rIL-2 on these cells. If there is a defect only in the IL-2-dependent pathway of IL-4 production, the addition of sufficient amounts of exogenous rIL-4 would be expected to restore the production of endogenous IL-4 because all Th2 T cells are responsive to high concentrations of IL-4.

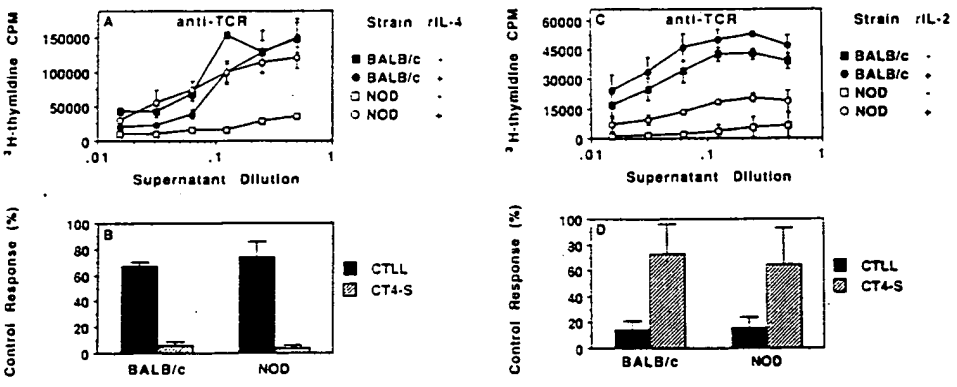


**Figure 5.** Exogenous rIL-4 completely restores IL-2 secretion by NOD thymic T cells whereas exogenous rIL-2 only partially restores IL-4 secretion by NOD thymocytes. (*A*) Thymic T cells from 8–12-wk-old NOD and BALB/c mice were activated by anti-TCR $\alpha/\beta$ in the presence of rIL-4 (125 U/ml). Culture supernatants were harvested and assayed for their IL-2 activity, as in Fig. 1, in the presence of the 11B11 anti-IL-4 mAb. The results of triplicate cultures are expressed as the mean ± SD, and are representative of three different experiments. (*B*) NOD- and BALB/c-stimulated T cell culture supernatants were assayed for their IL-2 and IL-4 activities in the presence or absence (control) of the 11B11 anti-IL-4 mAb. The results of triplicate cultures are expressed as the mean ± SD, and are representative of two different experiments. (*C*) Thymic T cells from 8–12-wk-old NOD and BALB/c mice were activated by anti-TCR $\alpha/\beta$ in the presence of rIL-2 (250 U/ml). Culture supernatants were harvested and assayed for their IL-4 activity, as in Fig. 2, in the presence of the S4B6 anti-IL-2 mAb. The results of triplicate cultures are expressed as the mean ± SD, and are representative of two different experiments. (*D*) NOD- and BALB/c-stimulated T cell culture supernatants were assayed for their IL-2 and IL-4 activities in the presence or absence (control) of the S4B6 anti-IL-2 mAb. The results of triplicate cultures are expressed as the mean ± SD, and are representative of two different experiments.

To test this possibility, BALB/c and NOD thymic T cell blasts were generated during an initial 24-h culture in the presence of anti-TCR $\alpha/\beta$ plus exogenous rIL-4 or rIL-2, and after washing and selection of viable T cell blasts, their profiles of IL-2 and IL-4 secretion were determined after a further 48 h of stimulation in the presence of only anti-TCR $\alpha/\beta$. NOD T cell blasts secreted significantly lower levels of IL-2 than did BALB/c T cell blasts (Fig. 6 *A*). Interestingly, BALB/c T blasts pretreated with rIL-2 or rIL-4 secreted equivalent levels of IL-2, and similar results were obtained for NOD T blasts. However, the levels of IL-4 secretion by NOD T blasts pretreated with rIL-2 or rIL-4 were considerably lower than those of BALB/c T cell blasts, and were equivalent to those observed in untreated control cultures (Fig. 6 *B*). Thus, although stimulation by anti-TCR plus rIL-4 restores the proliferative responsiveness of NOD T cells (Fig. 3) by enhancement of IL-2 secretion by these cells (Fig. 5 *A*), these data indicate that rIL-4 must be present continuously throughout the culture period so that normal IL-2 secretion by activated NOD T cell blasts can be achieved. In addition, these observations demonstrate that Th2 cells are significantly more unresponsive to both IL-2 and IL-4 than Th1 cells. Presumably, the continuous presence of IL-4 is required for NOD Th1 cell blasts to synthesize a sufficient amount of IL-2 that will enable these cells to progress through the cell cycle and proliferate. These results may also account for our previous findings that NOD thymic T cell blasts, generated after 3 d of culture in the continuous presence of rIL-2, are deficient in their TCR-mediated activation of $p21^{ras}$ and tyrosine phosphorylation of $p42^{mapk}$ (32). Because reduced $p21^{ras}$ and $p42^{mapk}$ activities inhibit progression to S phase of the cell cycle, these deficiencies likely mediate the proliferative unresponsiveness of these cells (32).

*In Vivo Administration of rIL-4 Protects NOD Mice against Diabetes.* Inasmuch as rIL-4 corrects the proliferative hyporesponsiveness of prediabetic NOD mice thymic T cells in vitro, we examined whether administration of murine rIL-4 in vivo prevents diabetes in NOD mice. As shown in Fig. 7, chronic treatment with the rIL-4 preparation supplied by Sterling Drug markedly suppressed diabetes development in NOD/Lt females (1/12 diabetic at the end of 20 wk vs. 9/12 receiving vehicle control). At 21 wk of age, widespread, severe insulitis was present in the pancreas of one of the two normoglycemic rIL-4-treated females examined. Severe insulitis was present in the pancreases of both of the normoglycemic vehicle-treated controls necropsied. Four of the nine rIL-4-treated mice remaining normoglycemic at the cessation of rIL-4 treatment at 20 wk subsequently developed diabetes by 52 wk of age. Of the five normoglycemic rIL-4-treated mice surviving to 52 wk, pancreases of three were free of insulitis, while insulitis was present in the other two. The single control mouse remaining normoglycemic at the end of 20 wk remained normoglycemic to 52 wk of age. However, only a single islet with granulated $\beta$ cells was found in the pancreas of this mouse, suggesting that diabetes was incipient. Heavy sialitis in submandibular glands and focal nephritis in the kidney was observed at 52 wk of age in all mice regardless of treatment.

A similar protective effect from diabetes was obtained in a second experiment in which another source of murine rIL-4 derived from a baculovirus expression system (18) was administered to NOD/Jd female mice beginning at 3 wk of age. This preparation, injected intraperitoneally for 15 wk in a similar amount (50 ng, twice weekly) to the rIL-4 used above in the first experiment, protected eight of eight NOD/Jd mice from diabetes upon analysis at 28 wk of age. The incidence of diabetes in untreated female NOD/Jd mice at this age was ~92%.

*Effect of In Vivo Administration of rIL-4 on Various Immune Parameters of NOD Mice.* The unusually high percentage of T cells present in the spleens of NOD/Lt mice (33) was not altered by treatment with rIL-4 (Sterling Drug preparation). (Table 3). In addition, cervical and pancreatic lymph nodes

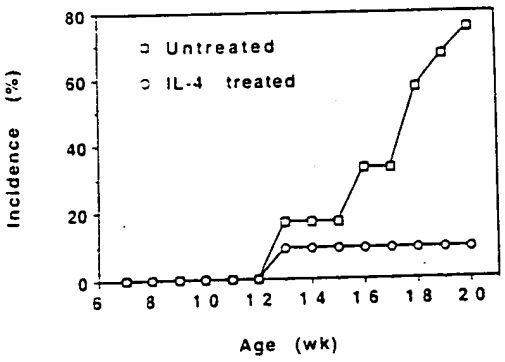


**Figure 7.** Decreased incidence of diabetes in female NOD/Lt mice treated in vivo with rIL-4. 12 female NOD/Lt prediabetic mice (randomized from four different litters) were injected twice weekly, from 6 to 20 wk of age, with 500 U (equivalent to 50 ng) murine rIL-4 or with vehicle (PBS + 1% serum from 6-wk-old prediabetic female NOD/Lt mice) only. Mice were screened weekly for the presence of glycosuria starting at 7 wk of age. Diabetes was diagnosed when mice were glycosuric for a consecutive 2 wk.

**Table 3.** *rIL-4 Treatment In Vivo Does Not Reduce the High Percentage of T Cells in NOD/Lt Spleen*

| Treatment | B cells | T cells | CD4 cells* | CD8 cells* | T/CD8 ratio |
|---|---|---|---|---|---|
| | % | % | % | % | |
| Vehicle | 31.5 | 52.4* | 35.4 | 17.0 | 3.0 |
| IL-4 | 36.3 | 53.8 | 38.1 | 15.7 | 3.4 |

Pooled splenic leukocytes from two normoglycemic females per group were enumerated by FACS® at the end of the 14-wk treatment period (i.e., at 20 wk of age).

* The percentage of T cells in the spleens of mouse strains at The Jackson Laboratory generally ranges from 25 to 30%. Thus, the presence of ~50% T cells in the spleens of NOD/Lt mice represents an unusually high percentage.

were enlarged in both rIL-4-treated and vehicle control-treated NOD/Lt mice (our unpublished data).

NOD/Lt macrophages secrete little IL-1 after LPS stimulation, and due to a stimulator cell defect, NOD/Lt T cells neither proliferate in response to self-MHC class II in an SMLR nor acquire immunoregulatory function (22). Since protection from diabetes in NOD/Lt mice treated with IL-2 in vivo was associated with increased IL-1 secretion from LPS-stimulated macrophages and a reversal of the SMLR defect (34), we examined whether these defects were also reversed by rIL-4 treatment. T cells from NOD/Lt female mice treated with rIL-4 from 6 to 20 wk of age failed to respond in an SMLR and did not acquire immunoregulatory function (Table 4). Treatment of NOD/Lt mice with rIL-4 in vivo also failed to increase appreciably the ability of macrophages from these mice to secrete IL-1 after LPS stimulation (Table 5). This suggests that the mechanism(s) by which the in vivo administration of rIL-4 protects prediabetic NOD mice from diabetes differs from that associated with rIL-2 therapy.

## Discussion

We have demonstrated that after activation by either anti-CD3 or anti-TCR $\alpha/\beta$ mAbs, IL-2 and IL-4 secretion by thymic and peripheral (splenic) T cells from NOD mice (>7 wk old) are significantly reduced. This suggests that NOD T cell proliferative unresponsiveness is mediated by a defect in the signaling pathway that links the TCR to IL-2 and IL-4 production. However, based on our studies of Con A activation of NOD thymocytes, a defect in IL-2-mediated signal transduction may not necessarily be causal to decreased thymic T cell proliferation for several reasons. First, equivalent percentages of NOD and BALB/c Con A–activated T cell blasts express HIL-2R both in the absence and presence of exogenous rIL-2. Second, the relative affinity ($K_d$) of HIL-2R for IL-2 and the number of HIL-2R molecules per cell on activated NOD and BALB/c thymic T cells are similar. This result differs from the previous report that HIL-2R expression is reduced about two- to threefold in mitogen-stimulated $CD4^+$ and $CD8^+$ spleen T cells from 10-wk-old NOD/ShiKbe mice, and that this reduction might mediate the pathogenesis of type I diabetes in these mice (25). T cell responsiveness in the thymus of NOD/Del mice therefore does not appear to be due to a decrease in either the level of expression or binding affinity of HIL-2R on $CD4^+$ or $CD8^+$ T cells. Third, Con A–activated NOD thymocytes secrete normal levels of IL-2. Fourth, although we did not formally test if IL-2-induced signal transduction is normal in thymic T cells from >7-wk-old NOD mice, we found that Con A plus exogenous rIL-2 induces an increase in HIL-2R expression. Hence, it seems that the post-HIL-2R part of the IL-2-mediated signaling pathway that results in this enhanced expression is intact.

Various modalities (anti-TCR mAbs vs. Con A) of NOD thymic and splenic T cell activation stimulated different levels of IL-2 secretion. The higher level of IL-2 secretion observed for Con A–activated thymic T cells may result from the ability of Con A to bind to and signal through several T cell surface molecules in addition to the TCR (35). This may not occur to the same extent for Con A–activated NOD splenic T cells, which yielded a low level of IL-2 secretion. There may exist different requirements of activation by NOD thymic and splenic T cells. Alternatively, Con A may activate a thymic T cell subpopulation that is absent from the spleen. Nonetheless, we observed that NOD thymic T cell unresponsiveness is maintained after activation by Con A even in the presence of normal levels of IL-2 secretion, HIL-2R expression, and IL-2/HIL-2R binding. This unresponsiveness is not restored

**Table 4.** *Treatment of NOD/Lt Mice with rIL-4 In Vivo Does Not Restore Their Ability to Activate Immunoregulatory T Cells in an SMLR*

| Strain | Treatment | SMLR response* | Suppression of MLC response‡ |
|---|---|---|---|
| | | *cpm ± SEM* | *%* |
| NOD | Control | 285 ± 165 | 0 |
| NOD | IL-4 | 524 ± 132 | 5 |
| SWR/Bm | Control | 3,320 ± 466 | 56§ |

Nylon wool–enriched T cells (SMLR responders) and irradiated splenic leukocytes (SMLR stimulators) were pooled from two mice each.

* SMLR blastogenic responses ($5 \times 10^5$ T cells cultured in triplicate with $5 \times 10^5$ stimulators) represent the mean [$^3$H]thymidine uptake over the final 8 h of a 6-d culture.

‡ Viable blast cells recovered on day 6 from bulk SMLR cultures ($5 \times 10^6$ T cells plus $5 \times 10^6$ stimulators) were added in triplicate at $2.5 \times 10^5$/well to an allogeneic MLC consisting of either $5 \times 10^5$ NOD or SWR T cells responding to $5 \times 10^5$ irradiated CBA/J splenocytes.

§ Significant suppression ($p < 0.05$ by Student's t test) from allogeneic MLC response in the absence of SMLR-activated T cells. The mean [$^3$H]thymidine uptake on day 4 for the unsuppressed MLC response of NOD T cells to CBA stimulators was 27,116 ± 3,329 cpm, whereas the response of SWR/Bm T cells to CBA stimulators was 69,064 ± 6,061 cpm.

**Table 5.** *Treatment of NOD/Lt Mice with rIL-4 In Vivo Does Not Increase the IL-1 Secretory Capacity of Macrophages*

| Macrophage source | Treatment | Endogenous IL-1 secretion | LPS-stimulated IL-1 secretion |
|---|---|---|---|
| | | *U/ml* | *U/ml* |
| NOD | Control | 0 | 1.3 |
| NOD | IL-4 | 0 | 2.0 |
| SWR/Bm | Control | 0 | 4.2 |

Peritoneal macrophages were pooled from two mice each and cultured at $10^6$/ml for 24 h in the presence and absence of 10 $\mu$g/ml LPS. IL-1 content of culture supernatants was determined by comparing their ability to support C3H/HeJ thymocyte proliferation stimulated by a murine rIL-1 standard (Hoffmann-La Roche, Inc., Nutley, NJ).

to normal by addition of physiological concentrations of exogenous rIL-2, and is at best only partially corrected by addition of supraphysiological amounts of rIL-2 (7; this report). A similar finding was reported for the inability of IL-2 to promote normal proliferative responses of Con A–stimulated NOD spleen T cells from 3- and 10-wk-old NOD/ShiKbe mice (25). These data are compatible with the observation that anergy can be induced in Th1 cells as a consequence of TCR occupancy by Ag in the absence of cell division (9). This can be achieved either because the Ag is presented by an APC that cannot provide the costimulatory signal(s) necessary for IL-2 production or because the T cell cannot respond to IL-2. The latter explanation likely accounts for NOD thymic T cell proliferative unresponsiveness, since we previously showed that NOD thymic APCs are capable of providing a costimulatory signal(s) and that, in comparison with control BALB/c thymocytes, NOD thymocytes proliferate relatively poorly in response to exogenous rIL-2 (7; this report).

Why then do NOD thymic T cells not respond well to IL-2? Is this due to the requirement of another proliferative stimulus? Since a determining factor in the control of T cell proliferation is the crosstalk between the IL-2 and IL-4 signaling pathways (28–30), and T cell production of IL-2 and IL-4 can be regulated by anergy induction (10), we considered that this additional stimulus might be IL-4, an autocrine growth factor for Th2 cells (31). We found that in vitro activated NOD thymic and splenic T cells fail to produce sufficient IL-4 to support their proliferation. Relatively little if any IL-4 secretion was detectable after stimulation of NOD thymic and splenic T cells by an anti-TCR $\alpha/\beta$ or anti-CD3 mAb. In addition, the level of IL-4 secretion by NOD thymic T cell blasts stimulated by anti-TCR plus either rIL-4 or rIL-2 for 24 h and then further activated for 48 h by anti-TCR was as low as that of control NOD T blasts. In contrast, their level of IL-2 secretion was slightly enhanced compared with that of control NOD T blasts. These findings indicate that IL-4 secretion by NOD thymic T cells is compromised to a greater extent than IL-2 secretion. Unlike rIL-2, exogenous rIL-4 completely restored the in vitro proliferative capacity of NOD thymic and splenic T cells. IL-4-induced restoration of the thymic T cell response stimulated by anti-TCR was associated with a normalization of the level of IL-2 secretion by these cells. This result agrees closely with the previous observation that IL-4 has a critical role in the stimulation of IL-2 production by mouse T cells in response to accessory cell–independent stimuli (plate-bound anti-CD3) (36). Similar results were also previously reported for the activation of human T cells by PHA (37) and anti-CD2 (38). Since IL-2 also potentiates the production of IL-4 by anti-CD3-activated T cells (18, 39), partial restoration of the in vitro proliferative response of NOD thymocytes by exogenous rIL-2 may arise from the inability of IL-2 to potentiate sufficient IL-4 secretion (Fig. 5 *C*). In addition, NOD thymocytes exhibit significantly reduced IL-4 secretion (Fig. 2 *B*) and diminished proliferative responsiveness after stimulation with anti-CD3 even in the presence of normal amounts of IL-2 (Fig. 4). For complete restoration of the NOD thymocyte proliferative response, IL-4 therefore needs to be present in addition to either exogenous rIL-2 or Con A–induced endogenous IL-2. Thus, decreased IL-4 secretion by activated NOD thymic and peripheral T cells appears to be a primary defect that elicits the proliferative unresponsiveness of these cells.

The in vivo administration of either rIL-2 (34) or two different preparations of rIL-4 (this report) protects prediabetic NOD mice against diabetes. Protection against diabetes in NOD mice by in vivo treatment with rIL-2 is associated with a reversal of the decreased splenic T cell SMLR of these mice and an increase in the LPS-induced IL-1 secretion by peritoneal exudate macrophages (34). In contrast, we report here that protection from diabetes by in vivo rIL-4 therapy is not associated with similar changes in the SMLR and/or IL-1 secretion. Thus, IL-2 and IL-4 appear to protect NOD mice from diabetes by different mechanisms. Unlike rIL-2, rIL-1, rIFN-$\gamma$, and rTNF-$\alpha$ (7), rIL-4 completely restores the in vitro NOD thymic T cell proliferative response and may achieve close to 100% protection from diabetes in vivo by a similar mechanism(s).

Reconstitution of lymphopenic prediabetic BB rats with the IL-4-producing $CD4^+CD45RC^{low}$ subset of Th cells but not with the IL-2-producing $CD4^+CD45RC^{high}$ Th subset protects these rats against autoimmune diabetes (40). In addition, diabetes and insulitis may be completely prevented by injection of the IL-2- and IL-4-producing subset of $CD4^+CD45RC^{low}TCR\ \alpha/\beta^+RT6^+$ thoracic duct T cells from healthy donors into a normal nonautoimmune rat strain that may be induced to become lymphopenic and diabetic by adult thymectomy and sublethal $\gamma$ irradiation (41). Altered ratios of $CD4^+CD45RA$ to $CD4^+CD45RO$ of PBL T cells together with decreased proliferative responses in the AMLR occur in human prediabetic patients, and may be diagnostic indicators of rapid progression to overt disease (42, 43). Immunohistochemical analyses performed using fluorescent mAbs indicate that IFN-$\gamma$ predominates in situ in the relative absence of IL-4 at the time of diabetes onset in NOD pancreatic islets previously transplanted beneath the kidney capsule of syngeneic female NOD mice at 4.5 mo of age. In contrast, IL-4 predominates and IFN-$\gamma$ is present in significantly lower amounts in NOD islets transplanted beneath the kidney capsule of control age-matched female NOD mice that were protected from diabetes by the previous administration (at 1 mo of age) of CFA (44). The in vivo administration of anti-IFN-$\gamma$ mAbs to NOD mice also prevents the onset of diabetes (45). Moreover, during the induction of tolerance, immunization with antigen and adjuvant induces an expansion of IL-4-producing Th2 cells (46). IL-4 inhibits the secretion of IFN-$\gamma$ by tolerant Th1 cells that retain the ability to secrete IFN-$\gamma$. In addition, Th2 cells that are expanded by IL-4 may secrete other cytokines, such as IL-10, which has also been shown to regulate Th1-dependent immune responses (46). Taken together, these observations suggest that T cell proliferative unresponsiveness in vitro and onset of diabetes in vivo may arise by the anergy and/or deletion of $CD4^+$ IL-4-producing Th2 cells in NOD mice, thereby enhancing the expansion of IFN-$\gamma$ producing potentially diabetogenic Th1 clones.

Mature $CD4^+CD8^-$ thymocytes are the predominant

IL-4 producers during primary immune responses (47). These IL-4-producing thymocytes are exported to the periphery and provide the IL-4 necessary for the generation of peripheral IL-4-secreting Th2 cells. Hence, the choice of an immature thymocyte to differentiate into a Th1 or Th2 mature T cell is likely dependent on the relative abundancy of IL-4 (47). In support of this notion is the observation that low doses of IL-4 are insufficient to promote IL-2-supported growth of thymic T cell precursors (27). A failure of NOD thymocytes to produce sufficient IL-4 for the differentiation of and export from the thymus of certain regulatory $CD4^-CD8^-$ Th2 clones may potentiate their unresponsiveness and eventual deletion. In this case, the balance between self-tolerance and autoimmunity would be disrupted and could result in type I diabetes.

Thus, IL-4 therapy may prevent the onset of diabetes in NOD mice by promoting the differentiation and exit from the thymus of "protective" regulatory $CD4^+CD8^-$ T cells. This protection afforded by IL-4 may also be mediated by its ability to perturb the development of $CD4^-CD8^-$ thymocytes, which was shown to occur in IL-4 transgenic mice in which the intrathymic expression of IL-4 was constitutively increased (48). Only peripheral $CD4^+$ T cells were found in significant numbers in these transgenic mice, while $CD4^-CD8^+$ thymocytes bearing high levels of TCR $\alpha/\beta$ were present in increased numbers, apparently because of their failure to emigrate to the periphery. Since these results indicate that IL-4 can regulate thymocyte maturation, they may explain in part how IL-4 can protect from diabetes by exerting reciprocal effects on the maturation of $CD4^+CD8^-$ (positive regulation) and $CD4^-CD8^-$ (negative regulation) thymic T cells.

In conclusion, our findings further document the therapeutic value of immunostimulation protocols for the prevention of autoimmune type I diabetes. In addition to IL-2 (34) and TNF-$\alpha$ (49, 50), we now report that IL-4 may also be used efficaciously for this purpose. Since IL-4 is required for the production of IL-2, this might explain in part why IL-2 deficiencies have been noted in both NOD (22, 51) and other strains of autoimmune mice (52), as well as in human type I diabetic patients (53). Further experimentation is required to test the possibility that prevention of type I diabetes in NOD mice by IL-4 is mediated by correction of an IL-2 deficiency.

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# Recombinant Human IL-10 Prevents the Onset of Diabetes in the Nonobese Diabetic Mouse

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The role of IL-10 in the pathogenesis of autoimmune diabetes mellitus was assessed in the nonobese diabetic (NOD) mouse. In these studies the effect of IL-10 was determined on three parameters of diabetes: The development of hyperglycemia, the development of insulitis, and the production of insulin by β cells. Initial experiments investigated the effect of anticytokine antibodies on the development of disease. These results indicated that monoclonal anti-IFN-γ antibody greatly reduced the incidence of hyperglycemia in female NOD mice, while anti-IL-4, IL-5, and IL-10 were ineffective. In subsequent studies, daily subcutaneous administration of IL-10, a known potent inhibitor of IFN-γ production by TH1 T cells, to 9 and 10-week-old NODs was shown to delay the onset of disease and significantly reduce the incidence of diabetes. Histopathology performed on pancreatic tissue demonstrated that treatment with IL-10 reduced the severity of insulitis, prevented cellular infiltration of islet cells, and promoted normal insulin production by β cells. Taken together these results indicate IL-10 suppresses the induction and progression of autoimmune pathogenesis associated with diabetes mellitus and suggest a potential therapeutic role for this cytokine in this autoimmune disease. 

## INTRODUCTION

Nonobese diabetic (NOD) mice (H-$2^{g7}K^dD^b$,I-$A^{g7}$) originally selected by Makino (1), exhibit a diabetic syndrome that strikingly resembles human type 1 autoimmune insulin-dependent diabetes mellitus (IDDM; 2, 3). Similar to its human counterpart, the murine disease is characterized by a progressive insulitis, where T lymphocytes and macrophages infiltrate and destroy the pancreatic islets through a cell-mediated autoimmune process that involves both $CD4^+$ and $CD8^+$ T lymphocytes (4–8). The onset and severity of disease in the NOD mouse is under genetic control (7–9) and appears to be influenced by hormonal factors since 70–80% of NOD females and only 20–30% of NOD males develop diabetes (10).

Presently, little is known about the role of cytokines in the pathogenesis of autoimmune diabetes. However, it is generally accepted that interleukin-2 (IL-2) and interferon-γ (IFN-γ) produced by $CD4^+$ T cells (11) contribute to the pathogenesis of IDDM. IL-2 may act to stimulate the growth of autoreactive T cells and IFN-γ is known to cause enhanced expression of class II MHC antigens (I-A antigens) by islet cells (12). The significance of the latter cytokine was illustrated by studies in which it was shown that transgeneic mice expressing IFN-γ under control of the human insulin promoter developed insulitis and diabetes (13), while similar IL-2 transgeneics developed insulitis but not diabetes (14). The contribution of IFN-γ to the diabetic pathogenesis is further substantiated by experiments demonstrating that administration of anti-IFN-γ antibody to NOD mice prevented the onset of diabetes (15). The role of other cytokines (IL-1 and TNF) in the development of autoimmune diabetes is not clear. Like IFN-γ, TNF-α enhances the expression of class II antigens on islet cells *in vitro* (12). However, unlike IFN-γ, TNF-α transgenic mice do not develop overt diabetes despite the presence of a pronounced pancreatic insulitis (16). TNF, as well as IL-1, exhibited cytotoxic activity against pancreatic β cells *in vitro* (17), but *in vivo* both cytokines have been shown to inhibit the development of autoimmune diabetes in NOD mice (18).

IL-10 is a TH2-derived cytokine which has been shown to have a variety of biological effects *in vitro*. It has the ability to inhibit cytokine production by human monocytes and murine macrophages. In addition, IL-10 was shown to suppress antigen-stimulated proliferation and cytokine production (IL-2, IFN-γ, TNF) by murine TH1 (CD4) T cells (19, 20). This latter activity suggests that one function of endogenous IL-10 is to downregulate cell-mediated immune responses, including autoimmune disease reactions, that are triggered in part by products of TH1 cells. We chose to test this hypothesis *in vivo* by evaluating the therapeutic efficacy of IL-10 in a spontaneous model of cellular autoimmune disease, the NOD mouse.

In this initial report, evidence is presented which demonstrates that development of autoimmune diabetes in NOD mice is suppressed following treatment with IL-10. Histopathological analysis of pancreatic

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tissue indicated that IL-10 reduced the severity of pancreatic insulitis and promoted normal levels of insulin production within the islets of Langerhans. The results suggest that downregulation of IFN-γ production may contribute to the mechanism by which IL-10 inhibits the onset of diabetes.

## MATERIALS AND METHODS

*Animals.* Eight-week-old female NOD mice were purchased from Taconic Farms Inc. (Germantown, NY) and were maintained and fed under pathogen-free conditions. The mice were fed irradiated Purina Certified Lab Diet (W-F Fisher and Son, Inc., Bound Brook, NJ) and provided with autoclaved water. The cages were changed frequently using irradiated Bed-O-Cob (W-F Fisher and Son, Inc.) bedding. Viral serology testing indicated that the NODs were free of Sendai, MHV, MVM, EDIM, PVM, SDAV/RCV, and KRV infections (Taconic Farms Inc.). Diabetic hyperglycemia occurs between 13 and 20 weeks of age in these NOD mice and the incidence of diabetes reaches 70–80% when the mice are 25–30 weeks of age. In this report all studies were initiated on animals that were 9–10-weeks old, an age at which all animals exhibit pancreatic insulitis.

*Assessment of diabetes.* The blood glucose level (BGL) was monitored weekly in all NODs by tail vein bleeds using a Glucoscan 3000 Blood Glucose Meter (Lifescan Inc., Mountainview, CA). Animals were considered to be diabetic when the BGL exceeded 200 mg/dL. Diabetic mice were continually monitored for BGL and usually died 4–6 weeks after developing diabetic symptoms.

*In Vivo anticytokine antibody treatment.* For *in vivo* studies, rat monoclonal IgG1 anti-mouse cytokine antibodies, including 11B11 (anti-IL-4) (21), 20F3 (anti-IL-6) (22), TRFK5 (anti-IL-5) (23), XMG1.2 (anti IFN-γ) (24), JES5-2A5 (anti-IL-10) (John Abrams, DNAX), and a rat IgG1 isotype control monoclonal antibody, GL113 (22), were prepared by Verax, Inc. (Lebanon, NH). These products were purified by ion exchange resin and Q-Sepharose chromatography to greater than 95% purity as assessed by HPLC. Endotoxin levels in the purified antibodies were <1.0 EU/mg protein. Lyophilized antibodies were diluted in endotoxin-free water (Sigma Chemical Co., Westbury, NY) and injected intraperitoneally into NOD mice twice weekly for 16–18 weeks.

*Recombinant human IL-10 (rHuIL-10).* Purified CHO-derived rHuIL-10 (sp act, $3 \times 10^6$ U/mg protein) was provided by the Department of Biotechnology, Schering-Plough Research Institute. For injections, rHuIL-10 was diluted from a frozen (−70°C) stock solution immediately prior to use.

*Treatment of NOD mice with rHuIL-10.* Groups of 9- to 10-week-old NOD mice (10 mice/group) were treated daily by the subcutaneous (sc) route with 1 μg of rHuIL-10 or a control protein, mouse serum albumin (MSA). In these studies, animals were monitored by weight and BGL until they were at least 25 weeks of age. BGLs were determined weekly and the diabetic incidence was recorded for each group. BGLs were always measured between the hours of 8–11 AM and rHuIL-10 injections were routinely administered in the afternoon.

*Histopathology.* The same small lobe of pancreas was removed from selected NODs in order to compare and contrast the pathology in the treatment groups. The two surviving diabetic MSA-treated (BGL 448–664 mg/dl) and three nondiabetic rHuIL-10-treated (BGL 86–159 mg/dl) NODs were chosen for this analysis 2 weeks after the last injection of rHu-IL-10. One piece of the tissue was fixed in 10% buffered formalin, embedded in paraffin, and sectioned for hematoxylin/eosin (H&E) staining. To detect insulin in pancreatic islet cells by histochemical techniques, a second portion of the pancreatic tissue was snap frozen in OCT compound (Tissue TCK, Miles Scientific) using liquid nitrogen/isopentane. Cryosections were cut 4 μ*M* thick, fixed, and stained with anti-insulin antibody (Dako Corp., Corpinteria, CA) using the immunoperoxidase technique described elsewhere (25). Similar analyses were performed on pancreatic tissue from nondiabetic 4 (BGL 88 mg/dl)- and 16 (BGL 156 mg/dl)-week-old NOD mice. The pancreatic tissues were prepared for histopathologic evaluation by Impath Laboratories (New York).

## RESULTS

*Effect of Anti-cytokine Antibodies on the development of diabetes in NOD mice.* Each of the anti-cytokine antibodies described above was injected intraperitoneally 2× per week at various doses (10, 100, and 200 μg/mouse) over a 16- to 18-week interval. BGL and diabetic incidence was monitored weekly. Administration of anti-IFN-γ was the only treatment that resulted in significant reduction in diabetic incidence when compared to untreated and isotype-treated controls. Treatment with anti-IL-4, anti-IL-5, and anti-IL-10 did not reduce the incidence of diabetes; however, at the highest dose (200 μg/mouse) anti-IL-6 antibody caused minimal reduction (from 70 to 50%) in disease incidence (data not shown). The therapeutic efficacy of anti-IFN-γ antibody as evidenced by a significant reduction in the incidence of diabetes and hyperglycemia in mice treated with this antibody is illustrated in Table 1. The maximum incidence of diabetes in the untreated control group was 70% with a mean BGL of 397 ± 69 mg/dl (Table 1). The disease incidence also

TABLE 1

Effect of Anti-IFN-γ (XMG1.2) on the Incidence of Diabetes in NOD Mice

| Treatment[a] | Incidence of diabetes[b] | BGL (mean ± SE)[c] mg/dl |
|---|---|---|
| None | 7/10 | 395 ± 69 |
| GL113 100 μg. | 7/10 | 384 ± 79 |
| XMG1.2 10 μg | 6/10 | 314 ± 81 |
| XMG1.2 100 μg | 2/10 | 156 ± 49[d] |

[a] NOD mice were injected intraperitoneally twice weekly for 15 weeks.

[b] Incidence at 25 weeks of age immediately following the last injection of antibody. NOD mice with consistent BGL measurements above 200 mg/dl were considered diabetic.

[c] Mean BGL ± SEM for each treatment group at 25 weeks of age following the last injection of antibody. Values were calculated using BGLs from diabetic and nondiabetic NODs ($n$ = 10).

[d] $P \leq 0.01$ when compared to untreated and GL113 controls.

reached 70% in the isotype-treated control group with a mean BGL of 384 ± 79 mg/dl. In comparison to the two control groups, treatment with anti-IFN-γ at a dose of 10 μg/mouse had no effect on the incidence of diabetes or on the BGL of the diabetic mice. However, in mice treated with 100 μg of anti-IFN-γ there was a dramatic decrease in disease incidence (from 70 to 20%) which was reflected in the mean BGL obtained for this group (156 ± 46 mg/dl; $P \leq = 0.01$) (Table 1). The incidence of diabetes and the mean BGL for the group of NOD mice treated with 200 μg anti-IFN-γ were similar to those observed in the group treated with 100 μg antibody.

*Effect of IL-10 on the development of autoimmune diabetes.* Two experiments were performed in which groups of female NOD mice, 9–10 weeks of age, received daily, subcutaneous injections of 1 μg of rHuIL-10 or an equivalent dose of a control protein, MSA. As shown in Table 2, treatment with rHuIL-10 greatly reduced the incidence of diabetes. Although in each experiment, MSA-treated control NODs exhibited overt diabetes by 4 months of age, none of the IL-10-treated mice had developed diabetes at this time point. There was no evidence of hyperglycemia in the IL-10-treated group until the animals reached approximately 5 months of age. When the data from the two experiments were combined, the incidence of diabetes was 85% (17/20) in the MSA control group and only 25% (5/20) for IL-10-treated NODs. Table 2 also shows the average BGLs obtained for the control and IL-10-treated groups in the two experiments. The MSA-treated NODs exhibited a mean BGL of 469 ± 46 mg/dl, while the IL-10-treated mice exhibited significantly reduced BGLs with a mean of 217 ± 50 mg/dl ($P \leq$ 0.001). The mean weight of the former was less than that of the IL-10 group, but these values were not significantly different at this time point (data not shown).

TABLE 2

Effect of rHuIL-10 on the Incidence of Diabetes in NOD Mice

| Treatment[a] | Incidence of diabetes[b] (months) | | | BGL (mg/dl)[c] after 15 weeks of dosing |
|---|---|---|---|---|
| | 4 | 5 | 7 | |
| 1 μg MSA | 3/20 | 8/20 | 17/20 | 469 ± 46 |
| 1 μg rHuIL-10 | 0/20 | 3/20 | 5/20 | 217 ± 50[d] |

[a] MSA and rHuIL-10 was initiated at 9–10 weeks of age and was continued for 15–16 weeks.

[b] NOD mice with consistent BGL measurements above 200 mg/dl were considered diabetic.

[c] Mean BGL ± SE for each treatment group. Values were calculated using BGLs from diabetic and nondiabetic NODs of each treatment group from two separate experiments ($n$ = 20).

[d] $P \leq 0.001$ compared to MSA controls.

*Effect of rHuIL-10 on the pancreatic histopathology of NOD mice.* A semiquantitative assessment of the effects of IL-10 treatment on the pathology that develops in the pancreas of NOD mice was determined by histological evaluation of tissue sections that included immunocytochemical detection of insulin. NOD mice were treated sc with MSA or rHuIL-10 (1 μg/mouse) for 17 weeks beginning at 10 weeks of age. Histopathological analyses were performed on pancreatic tissue removed from NOD mice in each treatment group in order to compare and contrast the resulting pathology. Therefore, two MSA-treated diabetic (BGL 448-664 mg/dl) and three IL-10-treated nondiabetic (BGL 86-159 mg/dl) NODs were selected for histological analysis 2 weeks after the final treatment. Cessation of treatment did not result in a change in the incidence f diabetes in either group at the time of sacrifice (2 weeks post-treatment). Pancreatic tissue from nondiabetic 4-week-old (BGL 88 mg/dl) and 16-week-old (BGL 156 mg/dl) NODs were also included in the study and served as control tissues for the detection of changes in insulitis and β cell function (insulin production). Table 3 summarizes the results from microscopic examination of pancreatic insulitis in various control and rHuIL-10-treated NODs. As can be seen, analysis of pancreatic tissue from untreated nondiabetic 4- and 16-week-old NODs indicated that in the former 100% of the islets were functional with virtually no leukocyte infiltrate, while in the latter more than 75% of islets exhibited leukocyte involvement and only 76% were functional (Table 3). Very few intact islets were observed in the pancreas of MSA-treated NODs and only 4% were found to be insulin positive. In contrast, analysis of pancreatic tissue from rHuIL-10-treated NODs showed that the majority of islets exhibited a peri-islet leukocyte infiltrate (80%), 9% were normal, and 90% were positive for insulin production.

Figure 1 shows representative results of histological analysis of sections of pancreas from control and IL-10-

TABLE 3

Presence of Insulitis in Control and rHuIL-10-Treated NODS

| Age (weeks) | No. of NODs | Treatment[a] | % Leukocyte-associated islets[b] | | | % Insulin (+) islets |
|---|---|---|---|---|---|---|
| | | | Normal | Peri-islet | Intraislet | |
| 4 | 1 | None | 100 | 0 | 0 | 100 |
| 16 | 1 | None | 14 | 64 | 22 | 76 |
| 29 | 2[c] | MSA | 0 | 12 | 88[d] | 4 |
| 29 | 3[e] | rHuIL-10 | 9 | 80 | 4 | 90 |

[a] Nods were untreated or treated with 1 μg of MSA or rHuIL-10 for 17 weeks.
[b] A minimum of 10–15 insets were counted per section.
[c] BGL 448 and 664 mg/dl for the two animals studied.
[d] No distinguishable islets were identified. This percentage based on areas of nodular lymphoplasmacytoid infiltrates where islets should have been visible.
[e] BGL range 86–159 mg/dl for the three animals studied.

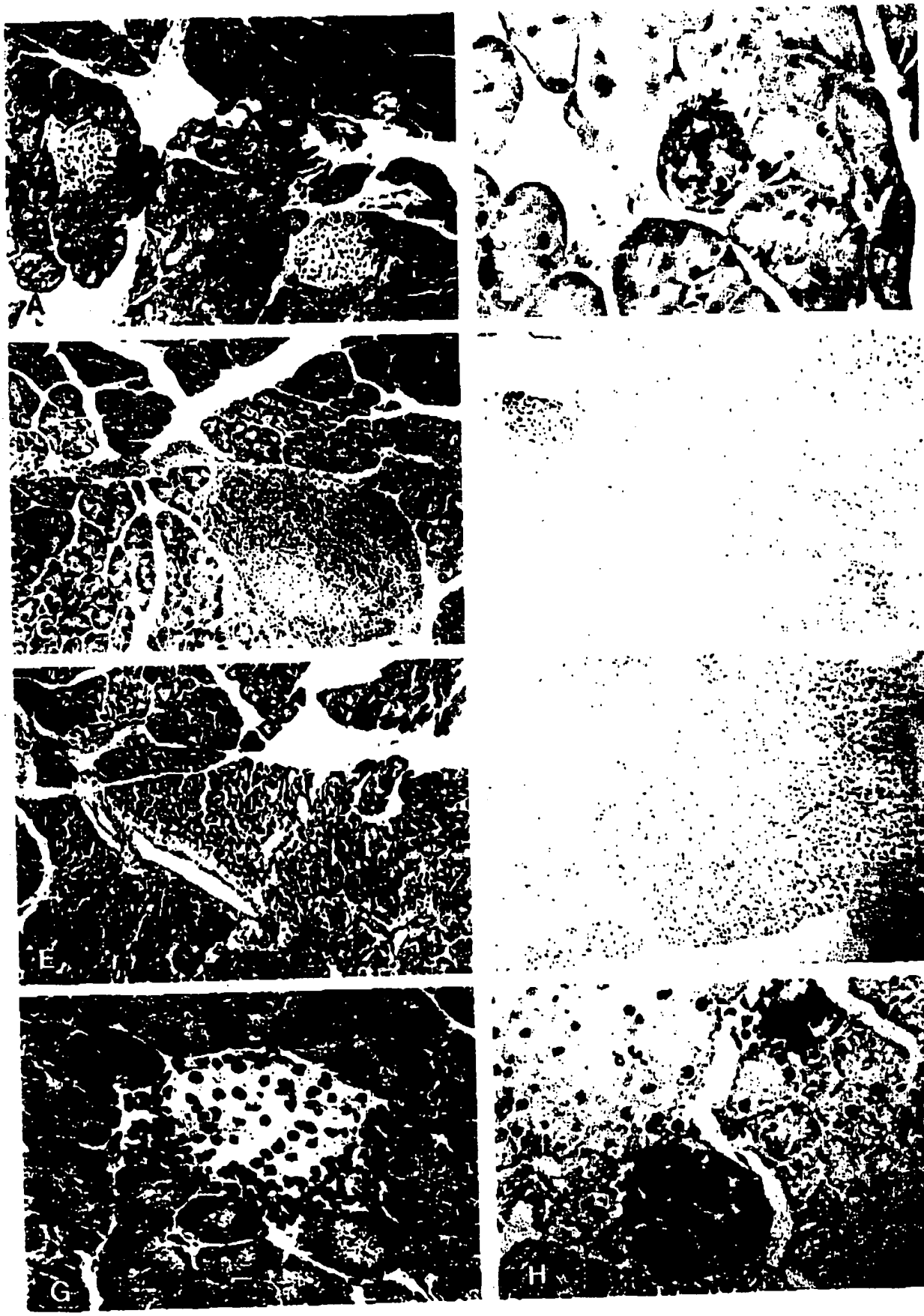
treated mice stained with H&E (Figs. 1A, 1C, 1E, and 1G) as well as results of immunoperoxidase staining for insulin (Figs. 1B, 1D, 1F and 1H). The histology of the pancreas from a 4-week-old nondiabetic female NOD mouse is shown in Fig. 1A. There were very few lymphocytes present among the pancreatic islets and the latter were essentially intact and readily distinguishable. Immunoperoxidase staining (Fig. 1B) revealed an abundance of insulin within the islet cells. Results of analyses performed on pancreatic tissue from a nondiabetic 16-week-old NOD mouse are illustrated in Figs. 1C and 1D. Two islets displaying different pathology are shown in Fig. 1C. One islet was small and comparable to those seen in the pancreas of the 4-week-old NOD. However, a second larger islet was obscured by an intense lymphoplasmatic infiltrate which extended into the lymphatic channel. Insulin could only be detected in the small relatively undisturbed islet (Fig. 1D). Results on pancreatic tissue from NOD mice treated with MSA for 17 weeks are shown in Figs. 1E and 1F. In this section there were no distinguishable islets by H&E staining (Fig. 1E) and nodular lymphoplasmacytoid infiltrates were observed in those areas where islets should have been visible (88%, Table 3). Insulin could not be detected following immunoperoxidase staining of this tissue (Fig. 1F). In marked contrast, H&E staining of pancreatic tissue from NODs treated with rHuIL-10 for 17 weeks (Fig. 1G) revealed intact islets, most of which were shown to be surrounded by a crown of lymphocytes (80%, Table 3). Despite the lymphocytic infiltrate, the general architecture of the pancreas and islets of Langerhans was similar to that observed in 4-week-old nondiabetic NODs. The intensity of the immunoperoxidase stain for insulin within cells of the pancreatic islets of IL-10-treated mice (Fig. 1H) was a further indication of the relatively normal architecture of the pancreas in these mice.

## DISCUSSION

The cytokine IL-10 was initially detected as a product of murine type 2 helper T-cells (TH2) that could suppress cytokine synthesis (e.g., IL-2 and IFN-γ) by activated cells of the TH1 subset. The capacity of IL-10 to suppress the production of TH-1-derived cytokines is a contributing factor to its modulatory effects on cell-mediated immune responses. Because of this property, it has been speculated that IL-10 may prove to be clinically useful in the treatment of autoimmune diseases; particularly those diseases where cell-mediated immunity to autoantigens predominates.

Human type 1 IDDM is an autoimmune disease in which IL-2 and IFN-γ appear to play a role in the onset and/or progression of disease. The NOD mouse is an experimental model of this disease where a progressive insulitis precedes the destruction of pancreatic islet cells as has been observed in the human counterpart. There are two reports in support of a role for IFN-γ in the development of diabetes in NOD mice. First, it has been demonstrated both *in vitro* and *in vivo* that in comparison to other mouse strains, NOD mice have an

FIG. 1. Comparison of pancreatic histology of untreated 4-week-old (A and B) and 16-week-old (C and D) NODs and NOD mice treated for 17 weeks with 1 μg of MSA (E and F) or 1 μg of rHuIL-10 (G and H) beginning at 10 weeks of age. Hematoxylin/eosin (H&E) staining and immunoperoxidase staining for insulin was performed on each tissue. Pancreatic tissue from 4-week-old mice stained by H&E (A, 100×) exhibited a normal pancreatic architecture with intact islets and few lymphocytes present in the area adjacent to the islet. Immunoperoxidase staining on this same tissue revealed normal production of insulin within the islet cells (B, 400×). A similar analysis performed on pancreatic tissue from a nondiabetic 16-week-old NOD mouse illustrated a large islet surrounded by a heavy lymphocytic infiltrate that had penetrated the lymphatic channel (C, 100×). Staining for insulin indicated low-level insulin production by a smaller undisturbed islet (D, 100×). By H&E staining there were no distinguishable intact islets in pancreatic tissue from MSA-treated 29-week-old NODs (E, 100×) and insulin was not detected in this tissue by immunoperoxidase staining (F, 100×). In contrast, intact islets were readily detectable in pancreatic tissue from rHuIL-10-treated 29-week-old NODs. Most of the intact islets from this tissue were surrounded (but not penetrated) by a small crown of lymphocytes (G, 400×). Cells within these islets also displayed intense staining for insulin (H, 400×).



nhanced capacity to produce IFN-γ (29). Second, IFN-γ transgenic mice develop an insulitis that precedes β cell destruction and the appearance of overt symptoms of diabetes (15). The data presented in this report are also consistent with the hypothesis that IFN-γ is a mediator of diabetes in NOD mice. Thus, the administration of an anti-IFN-γ antibody to NOD mice during that period when symptoms of diabetes usually become manifest prevented the onset of disease in the vast majority of treated animals. Moreover, IL-10, whose biological properties include the ability to inhibit FN-γ production by TH1 cells, was equally effective in preventing the onset of diabetes in NOD mice. The majority of NOD mice given a 16–17 week course of therapy with rHuIL-10 (1 μg/mouse) beginning at 9–10 weeks of age never developed disease. These mice remained disease free following cessation of therapy. Preliminary data indicates that the capacity of IL-10 to suppress disease is dose-related (0.1 μg/mouse is ineffective) and that its ability to prevent diabetes is not enhanced by starting therapy earlier (5–6 weeks of age) but is greatly diminished when therapy is initiated as late as 13 weeks of age (data not shown).

Histopathologic analyses of pancreatic tissue from IL-10-treated and MSA control animals provided additional insight into the mode of action of IL-10 in preventing the development of diabetes. Very few intact islets were evident on histological examination of pancreatic tissue from control NODs. A heavy cellular infiltrate was present and insulin could not be detected by immunoperoxidase staining of frozen sections of this tissue (Figs. 1E and 1F). In contrast, the pancreas of IL-10-treated NODs exhibited moderate insulitis (peri-islet infiltrate) and normal pancreatic architecture, including intact islets of Langerhans. In general, despite the more intense leukocyte infiltrate, the histology of the pancreas of IL-10-treated mice was similar to that observed in the pancreas of the 4-week-old NOD. Although the islets of IL-10-treated mice remained relatively intact and exhibited detectable insulin production, they were surrounded but not infiltrated by a crown of leukocytes (Figs. 1G and 1H). Likewise, it has been shown by others (27) that transgenic expression of IL-10 in the islets of Langerhans resulted in a pronounced pancreatic inflammation without β cell destruction and development of diabetes (27). Taken together, these observations indicate that IL-10 does not act by inhibiting the influx of inflammatory cells (lymphocytes and macrophages) into the pancreas. Rather it appears that *in vivo* IL-10 alters the local environment of the pancreatic islet in such a way that activation of those lymphocytes and inflammatory cells residing in the vicinity of the islet is prevented. This hypothesis is supported by *in vitro* studies (28) in which IL-10 was shown to significantly reduce antigen specific T cell proliferation and cytokin production by effectively downregulating th class II MHC expression on the antigen-presenting monocytes. Furthermore, attempts by our laboratory to detect circulating levels of cytokines in NOD mice during the onset and progression of diabetes have been unsuccessful (data not shown), strongly suggesting that locally produced cytokines contribute to the pathogenesis of diabetes. If results from further studies demonstrate the potential of IL-10 to downregulate the activation- and cytokine-producing capacities of leukocytes infiltrating the pancreas of NOD mice, then the relevance and clinical utility of IL-10 in the prevention of IDDM should be considered. Since it is likely that the prediabetic patient population selected for participation in clinical trials will have some degree of insulitis, an agent like IL-10, which in addition to restricting (further) cell migration into the pancreas also inhibits the disease-provoking activities of the cellular infiltrate, may prove to be therapeutically effective in the treatment of human IDDM.

In summary, this report describes results which indicate that IL-10 can prevent onset of diabetes in the NOD mouse which is known to have an autoimmune etiology. The data suggest that the cytokine synthesis inhibitory activities of IL-10, particularly its ability to inhibit IFN-γ synthesis by helper T cells of the Th1 subset, contributes to its efficacy in the NOD mouse model. Experiments are in progress to more precisely define the mechanism by which IL-10 prevents diabetes in the NOD mouse. These studies include quantitative analysis and phenotypic characterization of cells infiltrating the pancreas by flow cytometry as well as attempts to detect cytokine and cytokine mRNA expression by the specific cell types comprising the infiltrate.

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# EFFECTS OF IN VIVO ADMINISTRATION OF ANTI-CD3 MONOCLONAL ANTIBODY ON T CELL FUNCTION IN MICE

## II. In Vivo Activation of T Cells

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**Anti-CD3 mAb are known to be both immunosuppressive and mitogenic to T cells in vitro. However, only immunosuppression has been observed after in vivo administration of these mAb. The present study demonstrates that T cell activation does occur after in vivo administration of anti-CD3 mAb to mice, evidenced by increased IL-2R expression on T cells, CSF secretion, and extra-medullary hematopoiesis in the spleen. These effects required multivalent cross-linking of the mAb, since $F(ab')_2$ fragments failed to induce them. However, the $F(ab')_2$ fragments did induce modulation of CD3/TCR from the surface of T cells, demonstrating that TCR modulation is not sufficient to induce activation. In addition, interaction of the TCR with either intact or $F(ab')_2$ fragments of the mAb led to increased expression of CD8 in vivo, suggesting that the $F(ab')_2$ fragments of anti-CD3 mAb might be capable of inducing a T cell to undergo some, but not all, of the changes involved in reaching a fully activated state. Further study of the activating effects of anti-CD3 mAb might increase the understanding of the mechanisms of in vivo T cell activation and might also be exploited clinically to stimulate T cell function in immunocompromised states and to enhance hematopoiesis in myelodysplastic disorders.**

mAb directed against the CD3 invariant proteins of the TCR complex have been shown to be immunosuppressive both in vitro and in vivo. In vitro studies have demonstrated that these mAb can completely inhibit antigen mediated CTL activity in humans (1). One such antibody, OKT3, is used as an immunosuppressant in the clinical setting to treat organ graft rejection (2-4). We have recently shown that a hamster mAb, 145-2C11, directed against the CD3ε chain of the murine TCR complex, can suppress skin graft rejection in mice and cause both short and long term T cell dysfunction (5). Thus, the in vitro immunosuppressive effects of anti-CD3 mAb appear to also occur in the in vivo environment. In addition to their ability to suppress T cell function, anti-CD3 mAb also have potent activating properties in vitro. Incubation of human T cells with OKT3 results in T cell proliferation and lymphokine secretion (6). Similarly, in vitro incubation of murine spleen cells with 145-2C11 induces proliferation (7) and lymphokine secretion (8) (J.A. Bluestone and R. Cron, unpublished observations). The present study was undertaken to determine whether this T cell activation could be observed after in vivo administration of anti-CD3 mAb.

### MATERIALS AND METHODS

*Animals.* C57BL/10 male mice between 8 to 12 wk of age were obtained from The Jackson Laboratory, Bar Harbor, ME. Athymic BALB/c mice and NIH Swiss mice were obtained from the National Institutes of Health small animal production facility.

*Production and purification of anti-CD3 mAb.* 145-2C11 mAb was obtained by growing hybridoma cells in an Acusyst P machine (Endotronics, Minneapolis, MN) and collecting supernatant. Antibody was then purified by 50% ammonium sulfate precipitation followed by gel filtration on an ACA 34 ultrogel column (IBF Biotechnics, Savage, MD). The CD nomenclature has been used in this manuscript to designate defined cell surface molecules including Lyt-2 (CD8), L3T4 (CD4), and T3 (CD3).

$F(ab')_2$ fragments of 145-2C11 mAb were prepared by incubating purified mAb with pepsin in a ratio of 1/100 pepsin to mAb, in citrate buffer, pH 3.9, for 4 h at 37°C. The solution was then normalized to pH 7 with 3 M Tris-HCl, pH 8.6, and dialyzed into borate buffered saline, pH 8.5. $F(ab')_2$ fragments were separated from intact antibody by gel filtration on ACA 34 followed by passage over a protein A column. Purity of $F(ab')_2$ fragments was indicated by their inability, in soluble form, to induce proliferation of spleen cells.

*In vivo treatment of mice.* For flow cytometry and CSF studies, mice were treated with either 400 μg of intact mAb or an equivalent molar amount (250 μg) of $F(ab')_2$, administered in 1 dose i.v. via the lateral tail vein. This dose was selected based on its ability to completely saturate surface CD3 in spleen and lymph node cells, as previously shown (5). For histologic studies, mice were injected i.p. with either 400 μg of 145-2C11 (anti-CD3) or a combination of 250 μl of GK1.5 (anti-CD4) (9) and 250 μl of 2.43 (anti-CD8) (10) ascites.

*Proliferation assays.* Proliferation was measured by plating $1 \times 10^5$ C57BL/10 spleen cells from treated or control animals plus $2 \times 10^5$ untreated irradiated C57BL/10 spleen cells as accessory cells in 96 well microtiter plates (Costar, Cambridge, MA) with or without the addition of HR-IL-2[5] at different dilutions. In some assays 25% 2.4G2 (anti-FcR) (11) hybridoma cell culture supernatant was added to block anti-CD3-mediated T cell function (12). Experimental points

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[5] Abbreviations used in this paper: HR-IL-2, human rIL-2; EMH, extramedulary hematopoesis; FCM, flow cytometry; ATG, anti-thymocyte globulin; TRA, Texas Red-streptaridin.

were performed in triplicate. After incubation at 37°C for 3 days, each well was pulsed with 1 μCi of [$^3$H]TdR for 1 h. [$^3$H]TdR incorporation per well was measured and averaged for each triplicate.

*CSF assays.* Mice were bled 1, 2, 3, 6, 18, or 24 h after injection of intact 145-2C11 or 3 h after injection of 250 μg of F(ab')$_2$. CSF assays were performed as previously described (13). Briefly, serum samples were diluted to a final concentration of 6% in 1 ml of DME medium supplemented with 25% horse serum and 0.5% Bacto agar (Difco Laboratories Inc., Detroit, MI). A total of $1 \times 10^5$ bone marrow cells from National Institutes of Health Swiss mice were placed in 1 ml of DMEM supplemented with 25% horse serum and 0.3% Bacto agar, which was then seeded on top of the solidified 0.5% agar bed. Each serum sample was assayed in duplicate. Cultures were incubated for 7 days, at which time bone marrow colonies were counted with an inverted microscope.

*Histology.* Mice were euthanized with $CO_2$. Spleens were removed, weighed, and fixed in 10% neutral buffered formalin or Bouin's fixative, embedded in paraffin, sectioned at 6 μ, and stained with hematoxylin-eosin.

*FCM analysis.* Mice were killed 2, 12, 18, or 72 h after injection of the mAb and spleen and lymph nodes were removed. Two-color FCM analysis was performed on a FACSII (Becton Dickinson, Mountain View, CA) as previously described (14). Cells were stained with the following FITC-coupled mAb: anti-CD3 (145-2C11), anti-CD8 (Becton Dickinson), anti-CD4 (Becton Dickinson), goat anti-hamster Ig (Kirkegaard & Perry Laboratories, Gaithersburg, MD). An anti-H-2K$^{bm10}$ (128-3-3) (15) FITC was used as a negative green control. The following biotin-conjugated mAb were used followed by TRA (Bethesda Research Laboratory, Gaithersburg, MD) counterstaining: anti-IL-2R (3C7 or 7D4) (16), anti-Thy-1.2 (Becton Dickinson). Two biotin-conjugated mAb, anti-human CD7 (3A1) (17) and anti-H-2K$^k$ (36-7-5) (18) were used as negative red controls. Two-color immunofluorescence data were displayed as contour diagrams in which log intensities of green (FITC) fluorescence were plotted in 64 channels on the x axis and the log intensities of red (Texas red) fluorescence were plotted on the y axis. Constant values of the percentage of total cell number on the z axis were selected to draw the rings or contours around peaks of cells correlating FITC and TRA fluorescence. Log fluorescence in channels was converted to millivolts based on a standardization of the FACSII with fluoresceinated beads.

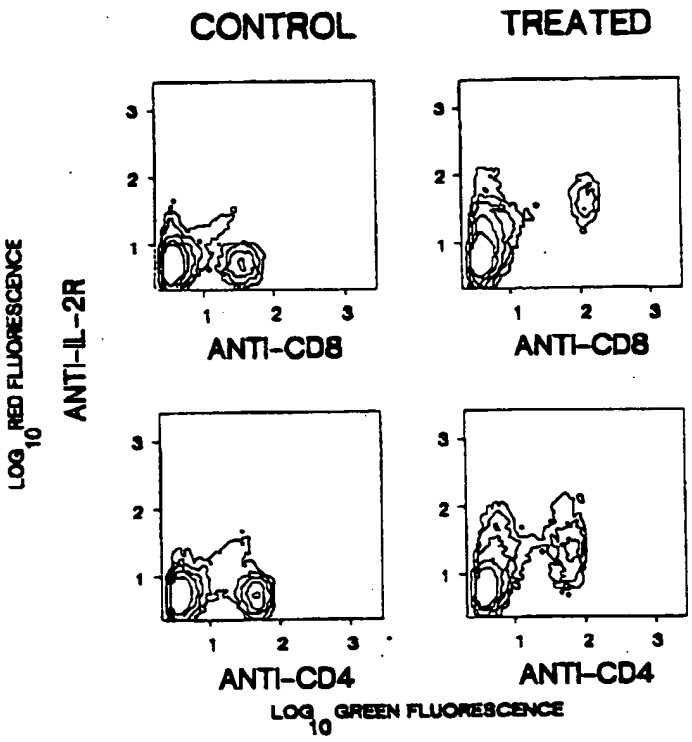


*Figure 1.* Increased IL-2R and CD8 expression 18 h post-injection of anti-CD3. Spleens were removed from control and treated mice and cells were examined by two-color FCM.

## RESULTS

*Anti-CD3 mAb induces in vivo IL-2 R expression.* The potential of the 145-2C11 mAb to activate T cells in vivo was evaluated by examining its ability to induce IL-2R expression after its administration. Spleen and lymph nodes were removed from treated animals at various times after injection of the mAb and analyzed by two-color FCM. Both CD8$^+$ and CD4$^+$ cells demonstrated an increase in IL-2R expression 18 h after treatment (Fig. 1). IL-2R expression was seen as early as 12 h after treatment and maximal expression, both in terms of intensity (data not shown) and cell number (Fig. 2) was observed 24 h after treatment. The IL-2R expressed on these in vivo-activated T cells was functional, because addition of exogenous HR-IL-2 in vitro resulted in high levels of proliferation compared to spleen cells from untreated animals (Fig. 3). This proliferation could not be blocked with an anti-FcR mAb (2.4G2), whereas proliferation to soluble anti-CD3 was completely blocked (Data not shown), indicating that the proliferation of the IL-2R$^+$ cells was a consequence of the added HR-IL-2 and not of residual anti-CD3 mAb present on the cell surface.

Despite the fact that significant depletion of both CD4$^+$ and CD8$^+$ cells is observed in the lymphoid organs of mice by 72 post-administration of 145-2C11 (5), the level of CD8 expression on non-depleted CD8$^+$ cells was found to be increased by 18 h post-administration. This increase in CD8 expression (397% in the experiment shown in Fig. 1) varied between 150 to 400% in multiple experiments. By 72 h after treatment, IL-2R expression (Fig. 2)

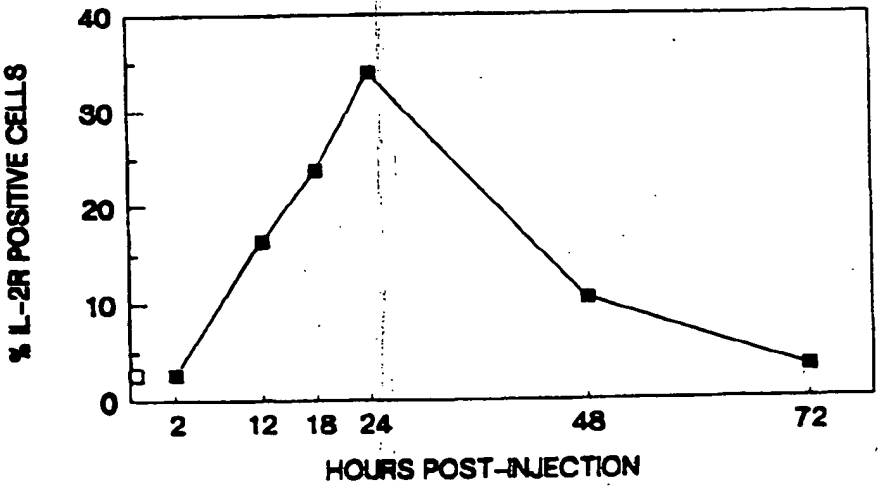


*Figure 2.* Time course of IL-2R expression after anti-CD3 treatment. Spleens were removed from control (□) and treated (■) mice and cells were examined by FCM for the presence of IL-2R. Percentage of spleen cells positive for IL-2R is plotted after subtraction of background levels of staining with control mAb (36-7-5). Each time point represents values for an individual animal with the exception of the 72-h point, which was a pool of spleen cells from five treated animals. These data were reproducible in multiple experiments.

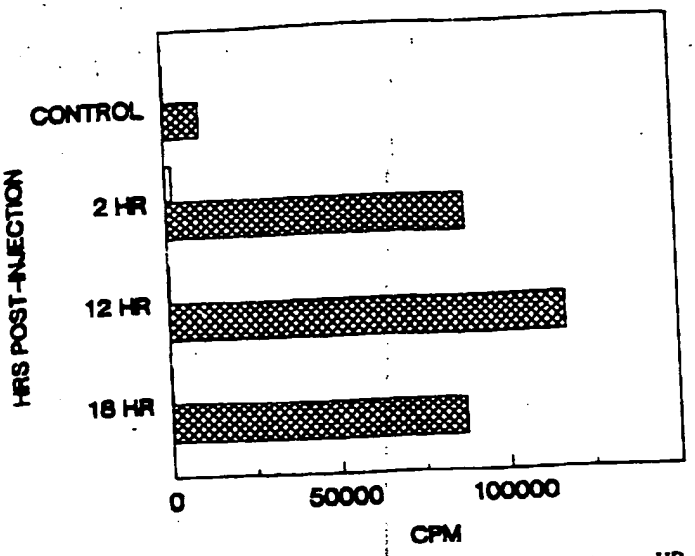


*Figure 3.* Proliferation of in vivo treated spleen cells to exogenous HR-IL-2. Spleens were removed at various times post anti-CD3 treatment and incubated in vitro with (*hatched bars*) or without (*open bars*) the presence of HR-IL2 (12.5 U/ml).

and CD8 expression (data not shown) had returned to normal, even though the mAb was still present in the serum of these mice (data not shown). The level of CD4 expression on resioval $CD4^+$ cells did not increase.

*Anti-CD3 mAb induces CSF secretion.* The previous results demonstrated that in vivo administration of anti-CD3 resulted in activation of T cells, evidenced by increased IL-2R expression and proliferation in response to co-stimulation with IL-2. Mice were next evaluated for possible physiologic effects of this T cell activation. Inasmuch as activated T cells can secrete various lymphokines, sera from anti-CD3 treated mice were assayed for the presence of CSF. C57BL/10 mice were injected i.v. with 145-2C11 and bled at various times post-injection. The sera were then analyzed for the presence of CSF. CSF was detected in 145-2C11-treated animals, but not in hamster Ig-treated animals (Fig. 4). CSF concentration in the serum after 145-2C11 injection peaked at 3 h post-injection, and was no longer detectable by 24 h post-injection (Fig. 4*A*). Doses as low as 10 μg stimulated CSF secretion 3 h post-injection of the mAb (Fig. 4*B*). Athymic B10 nude mice, which lack T cells, failed t produce CSF after injection of anti-CD3 (data not shown). Thus, it appeared that anti-CD3 was stimulating CSF secretion in vivo, presumably as a consequence of activating T cells.

*Anti-CD3 mAb induces EMH.* Inasmuch as a major effect of CSF is induction of hematopoiesis, mice treated with the anti-CD3 mAb were examined histologically for evidence of hematopoiesis. Peripheral blood from mice treated with the mAb showed an absolute neutrophilia with increased numbers of immature granulocytes beginning one day after injection and peaking 3 days after injection of the mAb (data not shown). In addition, significant EMH was observed in the spleen between 4 and 10 days after injection of the mAb, consisting of increased numbers of megakaryocytes and granulocytic and erythroid precursors in the parafollicular red pulp (Fig. 5*D*) and a doubling of splenic weight (Table I). For instance, on day 4 control and treated animals had total weights between 25.0 to 25.7 g in the controls and 22.5 to 24.4 g in the treated animals. However, spleen size in control animals was between 84 to 96 mg, whereas in treated animals it had increased to between 201 to 218 mg. To determine whether the observed EMH was a non-specific effect of T cell depletion or a direct result of T cell activation with subsequent release of hematopoietic growth factors, mice were treated with either anti-CD3 or with a combination of anti-CD4 and anti-CD8. Both treatments resulted in similar T cell depletion (80% on day 7 in anti-CD4 plus anti-CD8-treated animals; 76% on day 10 in anti-CD3-treated animals). However, significant EMH was observed histologically in spleens of anti-CD3-treated animals on days 4 (data not shown) and 10 Fig. 5 *C* and *D*), whereas anti-CD4 plus anti-CD8-treated animals showed no EMH on days 4 (data not shown) and 10 (Fig. 5*B*). Spleen size correlated with the histologic observations because anti-CD3-treated animals had a doubling in spleen size on days 4 and 10 compared to control animals, whereas anti-CD4 plus anti-CD8-treated

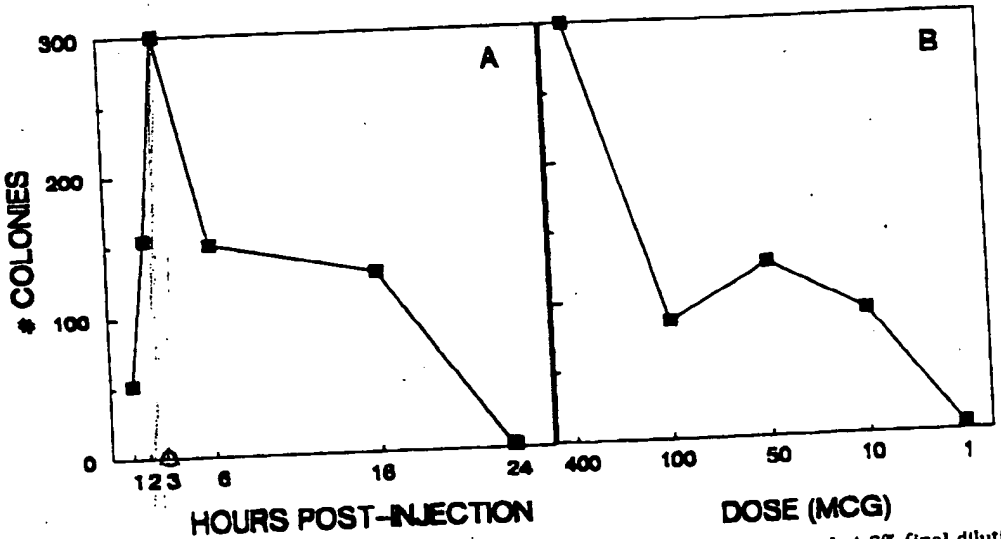


*Figure 4.* CSF in serum of mice after injection of anti-CD3. Pooled sera from three animals were placed at 6% final dilution with murine bone marrow cells, and the number of colonies were counted after 7 days. Each sample was tested in duplicate and the results averaged. In all cases, duplicate values differed by no more than 5%. *A*, Mice received 400 μg anti-CD3 (■), 400 μg hamster Ig (Δ), or 250 μg $F(ab')_2$ (○). (The number of colonies at 3h for anti-CD3 treated mice was more than 300.) *B*, Number of colonies after various doses of anti-CD3. Serum was collected 3 h after injection.

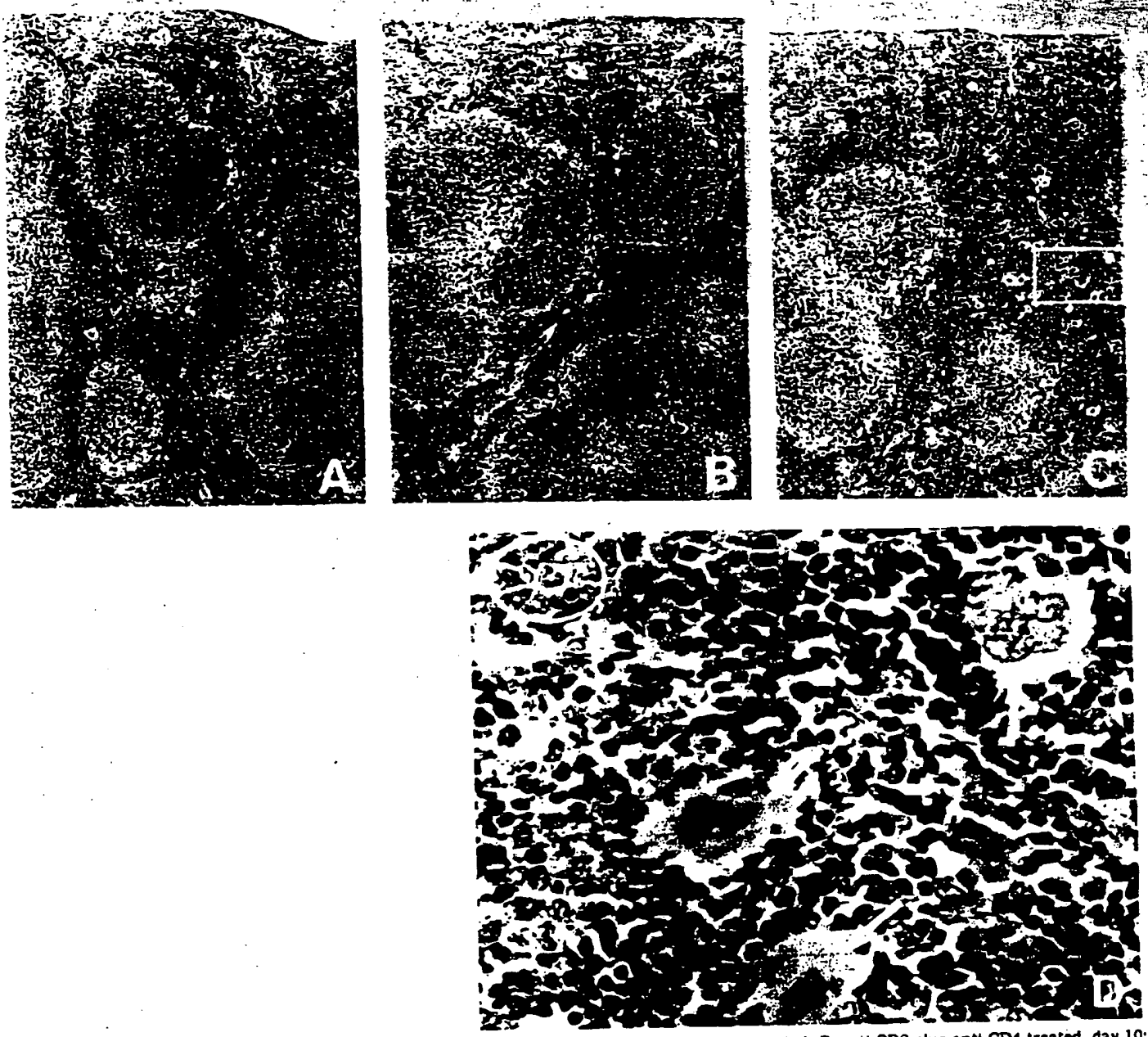


*Figure 5.* EMH after administration of 145-2C11. Representative splenic sections from *A*, untreated; *B*, anti-CD8 plus anti-CD4 treated, day 10; and *C*, anti-CD3 treated, day 10. Note the increased cellularity of the red pulp (area between follicles) in *C* (62×). *D*, enlargement of area within *inset* in *C*. Red pulp contains large numbers of hematopoietic cells, including megakaryocytes (*arrows*) and granulocytic precursors with characteristic "doughnut-shaped" nuclei (*circled area, upper left*) (×500).

animals showed no significant change in spleen size (Table I). Thus, the observed EMH after anti-CD3 treatment appeared to be a specific effect of the anti-CD3 mAb and not simply a consequence of depletion of T cells.

*F(ab')₂ fragments of anti-CD3 fail to activate T cells.* Previous in vitro studies with anti-CD3 mAb have suggested that multi-valent cross-linking of the mAb, either with accessory cells or by immobilization on a solid matrix, was necessary for delivery of an activation signal (19) (O. Leo and J.A. Bluestone, unpublished data). To determine whether this was also true in vivo, we prepared $F(ab')_2$ fragments of 145-2C11 by pepsin digestion. These $F(ab')_2$ fragments, in soluble form, did not induce in vitro proliferation of spleen cells, but were capable of inducing proliferation if immobilized on plastic (data not shown). The $F(ab')_2$ fragments were shown to bind specifically to the CD3 ligand, because they could block binding of FITC-coupled 145-2C11, as measured by FCM staining (data not shown).

As previously shown (Fig. 1), administration of intact mAb to mice resulted in increased IL-2R expression (Fig. 6*B*) at 18 h. Intact mAb also caused modulation of CD3/TCR 18 h after injection. This is demonstrated by two-color flow cytometry studies in which cells were pre-incubated with 145-2C11 and then stained with FITC-coupled goat anti-hamster Ig followed by bi tin-conjugated anti-Thy-1. In this manner T cells, which ar Thy-1⁺, can be distinguished from non-T cells, which are Thy-1⁻. Comparison f Fig 6 *D* and *E* demonstrates that after pre-incubation with 145-2C11, Thy-1⁺ cells from

TABLE 1

*Increased splenic size after in vivo anti-CD3*[a]

| | Control | | Anti-CD4 + Anti-CD8 | | 145-2C11 | |
|---|---|---|---|---|---|---|
| | | | Wt | | | |
| | Animal (g) | Spleen (mg) | Animal (g) | Spleen (mg) | Animal (g) | Spleen (mg) |
| Day 4 | 25.7 | 96 | 21.9 | 84 | 24.4 | 204 |
| | 25.0 | 84 | 25.5 | 109 | 23.5 | 218 |
| | | | 25.7 | 117 | 22.5 | 201 |
| Day 10 | 24.3 | 72 | 27.6 | 64 | 28.5 | 146 |
| | 28.4 | 86 | 27.9 | 86 | 24.1 | 172 |
| | | | 22.8 | 63 | | |
| Thy-1⁺ cell depletion | | | 80% (day 7) | | 76% (day 10) | |

[a] Increase in splenic weight 4 and 10 days after administration of 145-2C11. Percent T cell depletion during the time period between days 4 and 10, as measured by FCM analysis, is included for reference.

anti-CD3-treated mice (*E*) have a lower intensity of staining with FITC-coupled goat anti-hamster Ig than do Thy-1⁺ cells from control mice (Fig. 6*D*). This indicates modulati n of CD3/TCR from the cell surfaces of T cells from anti-CD3-treated mice. Injection of the $F(ab')_2$ fragments also led to modulation of CD3/TCR 18 h after injection, but no increase in IL-2R expression was observed (Fig. 6, *F* and *C*), despite complete saturation of CD3 on the cell surface as measured by FCM analysis (data not shown). In addition, no CSF was detected in the serum after injection of $F(ab')_2$ fragments (Fig. 4, *open circle*). CD8 expression after $F(ab')_2$ treatment was somewhat variable in multiple experiments, ranging from no detectable increase to a three-fold increase over control animals. In the experiment shown in Figure 6*C*, CD8 expression increased by 150%. Thus, intact mAb appeared necessary to induce IL-2R expression, but was not needed to induce modulation of th TCR and increased CD8 expression.

## DISCUSSION

Anti-CD3 mAb induce rapid suppression of T cell function in vivo, and are used clinically to suppre s organ graft rejection. However, in vitro studies have shown that these mAb can also be potent activators of T cells. Therefore, we evaluated one such mAb, 145-2C11, for its ability to induce in vivo T cell activation. The present study demonstrates that an anti-CD3 mAb can have dramatic activating effects in vivo, including induction of IL-2R, CSF secretion, and EMH. As has been shown in vitro, this in vivo activation appears to require multi-valent cross-linking of the mAb via its Fc portion. $F(ab')_2$ fragments of the mAb failed to induce IL-2R expression r CSF production. Experiments designed to directly test the in vivo requirement for multi-valent cross-linking by administration of anti-FcR mAb (2.4G2) before administration of intact anti-CD3 were inconclusive, perhaps due to other FcR in the mice which are able to cross-link the 145-2C11 mAb. Of interest is that the $F(ab')_2$ fragments did induce modulation of the CD3/TCR complex. Inasmuch as, in this study, CD3/TCR modulation occured without induction of IL2-R, it is clear that modulation is not sufficient for transmission of a complete activating signal.

Both the intact mAb and the $F(ab')_2$ fragments induced

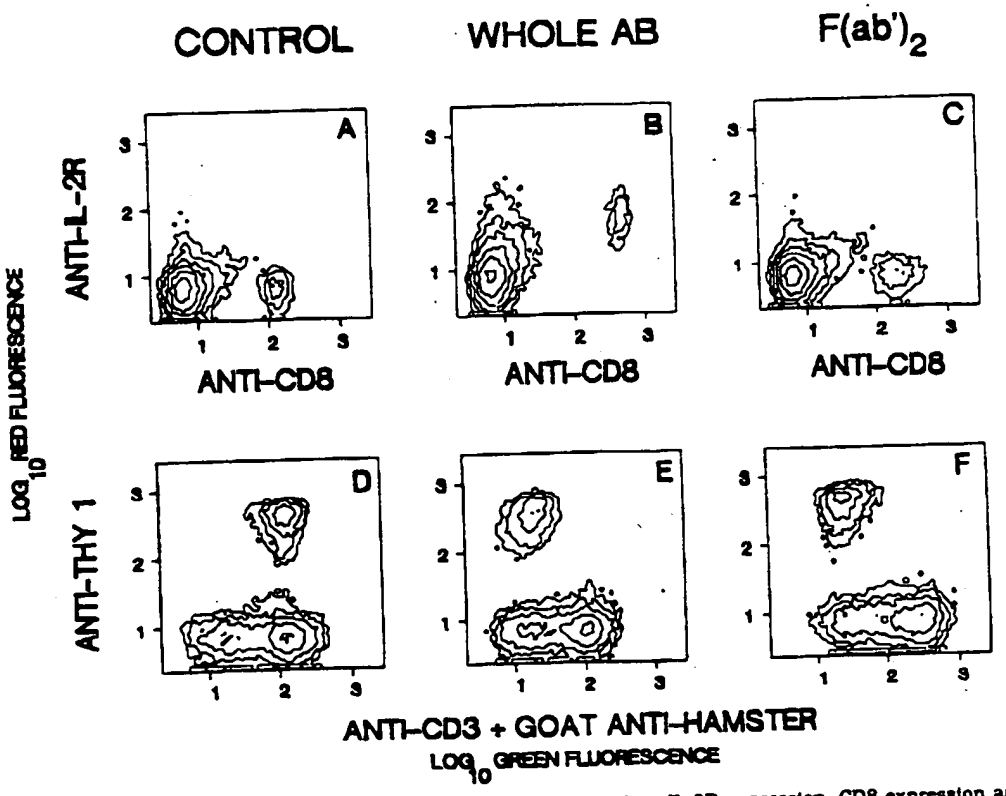


*Figure 6.* Comparison of ability of intact and $F(ab')_2$ fragments of anti-CD3 to induce IL-2R expression, CD8 expression and TCR modulation. Spleen cells from two control, two whole mAb-treated, and two $F(ab')_2$-treated animals were removed 18 h after treatment, pooled, and stained by two-color FCM. Modulation after whole or $F(ab')_2$ anti-CD3 treatment is indicated by decreased staining of Thy-1⁺ cells with goat-anti-hamster FITC after incubation with 145-2C11 (*D* and *E*) compared to control (*C*). Note that the goat anti-hamster Ig reacts with murine B cells, but T cells are distinguished from B cells by staining positively with the anti-Thy-1 mAb.

increased levels of CD8 expression in vivo. This increase in CD8 expression after in vivo modulation of the CD3/TCR complex is in contrast t in vitro observations both with 145-2C11 (R. Hirsch and J.A. Bluestone, unpublished data) and with other anti-CD3 mAb (20) in which CD8 appears to co-modulate with the TCR. Whereas CD8 may play a role in formation of conjugates between $CD8^+$ T cells and APC or target cells by interacting with class I MHC Ag, recent evidence suggests that CD8 might be directly involved in T cell activation because anti-CD8 mAb can inhibit TCR-mediated cytolysis in the absence of target cells expressing MHC class I Ag (12, 21). In addition, transfection of human CD8 into a murine T cell hybridoma increases IL-2 production when the hybridoma is exposed to cells expressing class I MHC (22). Thus it appears that the CD8 molecule may play a role in T cell activation. The increased CD8 expression seen after in vivo anti-CD3 treatment may facilitate T cell activation. It is possible that the increased CD8 expression seen after in vivo administration of $F(ab')_2$ fragments of anti-CD3 reflects a state of partial activation. If this is true then the study of these cells might increase the understanding of the mechanisms of T cell activation.

Although the 145-2C11 mAb clearly induced activation of T cells in vivo, the effects appear to be non-specific in that all T cells were affected. In addition, the activating effects were overshadowed by the immunosuppressive ones because the dose used in this study causes modulation of CD3/TCR and depletion of T cells. Thus, such a high dose would probably not be useful for enhancement of specific immune responses requiring activation of clones of T cells against a single target pathogen or tumor. However, the results of this study suggest that anti-CD3 mAb may be useful in increasing overall immune function in immunocompromised states if used in a dose that would activate T cells without causing depletion or CD3/TCR modulation. Ongoing efforts are being devoted to finding regimens that induce IL-2R expression without detectable TCR modulation or T cell depletion, enabling the separation of the immunopotentiating effects of anti-CD3 mAb from their immunosuppressive ones. This may provide a means to stimulate immune function in circumstances in which T cell function is compromised.

In light of the results presented herein, it would be of interest to examine serum from patients within the first few hours after administration of OKT3 for the presence of CSF. Release of lymphokines after T cell activation might explain some of the early side effects associated with OKT3 administration. In addition, the possibility of using anti-CD3 mAb to physiologically increase CSF production might find a variety of uses. For instance, granulocyte-macrophage CSF has been found to be effective in increasing granulocyte counts in patients infected with the HIV virus (23) and in myelodysplastic disorders (24). Also, ATG is used clinically to treat aplastic anemia. Although it has been postulated that ATG stimulates hematopoiesis due to its immunosuppressive properties (25), the results of the present study suggest that the effects may be secondary to activation of T cells with subsequent release of CSF. Inasmuch as ATG is a polyclonal preparation of antibodies, it may contain antibodies specific to T cell surface Ag which can mediate an activating signal, including anti-CD3/TCR antibodies. In this regard, ATG has recently been shown to stimulate CSF production from monocytes in vitro (26, 27). Thus, anti-CD3 mAb in non-immunosuppressive doses might be useful as a stimulus for hematopoiesis.

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# ANTI-CD3 ANTIBODIES INDUCE T CELLS FROM UNPRIMED ANIMALS TO SECRETE IL-4 BOTH IN VITRO AND IN VIVO[1]

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**Recently, functional heterogeneity among Th cells has been recognized. Based on pattern of lymphokine secretion, two mutually exclusive subsets of CD4+ cells have been defined and designated Th1 (secreting IL-2 and IFN-γ) and Th2 (secreting IL-4 and IL-5). Identification of these subsets was mostly based on the study of long term cultured T cell lines and clones, and little is known about the Th heterogeneity in vivo. In particular, it has been suggested that IL-4 producing cells cannot be detected in vivo or in primary stimulations in vitro unless responder cells had been previously primed. Our data however, indicate that anti-CD3 mediated stimulation can induce T cells isolated from unprimed animals to IL-4 production. An assay system based on the ability of IL-4 to increase Ia expression of B cells present in the environment of activated T cells was found to be more sensitive than detection of secreted IL-4 in the supernatant by conventional bioassays and was used to study IL-4 production by unprimed lymphocytes polyclonally stimulated in vivo and in vitro by anti-CD3 mAb. The results obtained indicate that CD4+ CD8- T cells able to produce IL-4 upon receptor-specific stimulation exist in the preimmune pool of adult animals. Remarkably, these cells can also be stimulated in vivo by treating animals with anti-CD3 mAb, as indicated by the in vivo induction of IL-4 specific mRNA and hyper-Ia expression on B cells. These results indicate that the inability to detect IL-4 in primary cultures is not due to different activation requirements of Th2 cells but may simply result from their lower frequency in unprimed animals.**

It is now well established that Th cells play a central role in the initiation of a specific immune response. The available evidence to date indicates that after Ag recognition, Th cells secrete a series of regulatory factors or lymphokines that have an effect on the activation, proliferation and function of T cells, B cells, and other cell types.

The analysis of functional phenotype and of pattern of lymphokine secretion has recently allowed the identification of discrete subsets of Th cells (1–4). Thus, based on the analysis of Th clones and cell lines, two mutually exclusive subsets of CD4+ cells have been recently recognized and designated Th1 and Th2 (4, 5). Th1 cells produce IL-2, IFN-γ, and lymphotoxins, whereas Th2 cells secrete IL-4 and IL-5 in response to the same stimuli (6). Although Th1 clones seem ideally equipped to act in cell-mediated immunity and Th2 to provide B cell help, in vitro analysis have shown that Th1 and Th2 clones perform both distinct and overlapping functions. For example, both subsets can help B cells to produce antibodies, although quantitative and qualitative differences exist (6–10). In contrast, only Th1 cells seem to be involved in delayed-type hypersensitivity (11).

The identification of these two subsets of CD4+ helper cells is mostly based on studies performed with tumor T cell lines, T cell hybrids, and Ag-specific T cell clones. It is therefore not clear whether these two subsets exist in vivo and represent distinct lineages of T cells. In particular, the frequency, tissue distribution and ontogeny of cells capable of secreting IL-4 in vivo are not known. These studies have been hampered by the fact that freshly isolated T cells from unprimed animals often failed to secrete detectable amounts of IL-4 and IL-5 when stimulated in vitro by lectins or alloantigens, whereas IL-2 was readily detectable in the same cultures (12, 13).

Our study was undertaken after the observation that soluble anti-CD3 antibodies induced IL-4 production by unprimed T cells, as reflected by an increased Ia expression on B cells present in the same microenvironment of activated T cells. This sensitive assay of IL-4 activity allowed us to probe the repertoire of T cells from unprimed animals and we found that in contrast to previously published reports, cells able to synthesize IL-4 are present in the preimmune lymphocyte pool.

## MATERIALS AND METHODS

*Mice.* DBA/2 mice were purchased from the Institute of Cellular Pathology, Brussels (Belgium); BALB/c mice were obtained from Olac (Bicester, England).

*mAb.* Culture supernatants from the following lines were used as source of antibodies: anti-CD3: 145-2C11 (14); anti-CD4: GK1.5 (15) and RL17.2 (16); anti-IL-4: 11B11 (17); control rat IgG1: LO-DNP-2, specific for the dinitrophenyl hapten (18); anti-$IA^d$: MKD6 (19) and 25-9-17 (20); anti-$IE^{k,d}$: 14-4-4S (21).

The mAb AH46 (IgA.k of BALB/c origin), specific for isotypic determinants on the light chain of the hamster mAb 145-2C11 was derived in our laboratory. $F(ab')_2$ and Fab fragments of the 145-2C11 mAb were prepared by cleavage of purified IgG by proteolysis

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using, respectively, pepsin (Boehringer Mannheim GmbH, FRG) and papain (Sigma Chemical Co., St. Louis, MO). Briefly, the mAb 145-2C11 was purified from culture supernatants by affinity chromatography over a column of protein A-Sepharose (Pharmacia, Uppsala, Sweden) and incubated with the appropriate enzyme according to standard procedures (22). After digestion and enzyme inactivation by dialysis, the preparation was purified by chromatography over protein A-Sepharose to remove the undigested material. The purified fragments were shown by gel electrophoresis and ELISA using a mAb specific for the Fc portion of the 145-2C11 (clone AH6, generated in our laboratory) to contain less than 1% of intact IgG.

*In vitro experiments.* Cellular proliferation and the expression of Ia determinants were evaluated after stimulation of splenocytes by anti-CD3 mAb.

Intact or enzyme-digested anti-CD3 mAb were added to the culture in their soluble form or insolubilized as follows. Culture plates were coated overnight at 4°C with an anti-L chain mAb (clone AH46, 100 μg/ml). After extensive washing, anti-CD3 antibodies or its fragments were added to the plates and incubated for 2 h at 37°C. Plates were then washed and spleen cells were added for culture. This method of insolubilization using an anti-hamster antibody was preferred to the direct adsorption of anti-CD3 mAb to solid phase after the finding that Fab fragments bound poorly to plastic trays. Cells were cultured at $1.5 \times 10^6$ cells/ml in 2 ml of complete media (RPMI 1640 supplemented with 10% FCS, sodium pyruvate, nonessential amino acids, glutamine, and 2-ME). The cultures were incubated at 37°C in 7% $CO_2$ in humidified air. When indicated, LPS (Difco, Detroit, MI) was added to the cultures at 6 μg/ml; CsA[3] (a kind gift of Sandoz Ltd., Basel, Switzerland), was added to the cultures at a final concentration of 1 μg/ml. rIL-4 was produced in methotrexate amplified Chinese hamster ovary cells and was a kind gift from Dr. W. Fiers (State University, Gent, Belgium). Intensity of Ia Ag expression was evaluated after 18 h of culture by flow cytometry as described below. For anti-CD3 induced proliferation, $2 \times 10^5$ splenocytes were cultured in 0.2 ml cultures for 3 days. 0.5 μCi of $^3$H-thymidine (Amersham International, Buckinghamshire, UK) was added to each well 18 h before collection of the cultures with an automated cell harvester.

*T cell subset analysis.* Spleen cells were depleted of $CD4^+$ or $CD8^+$ lymphocytes by C′-mediated lysis followed by the addition in culture of antibodies to CD4 or CD8 to functionally block cells that might have escaped lysis. Briefly, splenocytes were incubated for 30 min at room temperature with saturating amounts of anti-CD4 mAb (clone RL17.2) or anti-CD8 mAb (clone 83-12-5, kindly provided by J. Bluestone, University of Chicago, Chicago, IL). Cells were then washed and incubated with C′-containing selected rabbit serum (produced in our laboratory) for 30 min at 37°C. Anti-CD4- and anti-CD8-treated cells were then cultured in the presence of, respectively, anti-CD4 (clone GK1.5) and anti-CD8 (clone 83-12-5) mAb (20% of culture supernatant from the corresponding cell lines).

*In vivo experiments.* DBA/2 mice were injected i.v. with a single dose of 25 μg of affinity purified anti-CD3 mAb. Control animals were injected with 25 μg of hamster Ig or saline. IL-4 production was evaluated by increased Ia expression on splenic B cells and by detection of IL-4 mRNA in spleen cells as described below.

Inhibition of IL-4 production was attempted by pretreating animals with s.c. injections of CsA (25 mg/kg in 90% olive oil/10% ethanol) 18 and 3 h before administration of anti-CD3 mAb. Blocking experiments with mAb were performed as follows. Mice received i.p. injections 24 and 1 h before anti-CD3 treatment with 1 ml of ascitic fluid containing 4 mg of rat $IgG_1$ Ig specific for IL-4 (mAb 11B11) or the hapten DNP (control group, mAb LO-DNP-2).

*Immunofluorescence.* Cells were suspended in PBS containing 0.5% BSA and 0.1% sodium azide (flow microfluorimetry media). Saturating amounts of antibody-containing culture supernatant were added to $10^6$ cells in 0.1 ml and incubated on ice for 30 min. Cells were then washed in flow microfluorimetry media and incubated for 30 min with biotin-coupled goat antibodies specific for murine IgG2a, before a further incubation with FITC-labeled streptavidin. Both reagents were purchased from Amersham International. Biotinylated 14.4.4S mAb was used in some experiments.

Cells were analyzed using a Cytofluorograph (Ortho Diagnostic Systems, Westwood, MA) (Figs. 1, 2, 6, and 10) or a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) (Figs. 3, 4, 5, 7, and 9). In all cases, cells were gated for size and side scatter to eliminate from analysis debris and dead cells. Fluorescence results are generally expressed as the mean channel fluorescence of the FITC-positive peak on an arbitrary log scale.

Two-color analysis for surface IgM and Ia Ag was performed by incubating cells with FITC-coupled goat anti-IgM antibodies (Cappel, Malvern, PA) and biotinylated anti-Ia mAb counterstained with phycoerythrin-conjugated streptavidin (Becton Dickinson).

*RNA isolation and Northern blots.* Total RNA was isolated from splenic cells of DBA/2 mice by guanidinium thiocyanate/cesium chloride method (23) followed by a phenol-chloroform extraction. Of total RNA 5 μg were denaturated in DMSO-Glyoxal, submitted to 0.01 M $NaH_2PO_4$ 1% agarose gel electrophoresis as described (24) and transferred to nylon membranes (Amersham International) by capillary blotting.

Filters were dried, baked for 1 h at 80°C, and the positions of 28S and 18S rRNA bands were determined by UV shadowing. The pSP65-mIL-4 9(A) plasmid contains the IL-4 cDNA cloned downstream from the SP6 promoter and was kindly provided by Drs. R. Devos and W. Fiers, Gent, Belgium. [$^{32}$P]RNA probes were transcribed using SP6 polymerase (Boehringer Mannheim).

Filters were hybridized with [$^{32}$P]RNA probes 24 h at 60°C in the prehybridization solution (10× Denhardt solution, 0.1% SDS, 200 μg/ml DNA salmon sperm, 5× SSC, and 50% formamide) then washed at 60°C in 2× SSC for 30 min followed by 0.2× SSC for 30 min before autoradiography at −80°C for 1 wk.

## RESULTS

*IL-4 production by unprimed T cells.* Our study was initiated because of the observation that in vivo administration of soluble anti-CD3 mAb induces a rapid increase of Ia expression on B cells. Although a number of stimuli have been described that increase the expression of B cell surface Ia, including cross-linking of surface Ig (25, 26) or mitogens (27), IL-4 is the only T cell-derived lymphokine known to date that is able to induce the majority of purified B cells to high expression of class II determinants (28, 29). We therefore postulated that IL-4 was secreted by naive T cells after TCR/CD3-mediated activation and developed an in vitro model to test this hypothesis. Spleen cells from unprimed DBA/2 mice were cultured in anti-CD3-coated plates for 18 h at 37°C. After culture, the production of IL-4 was monitored by two independent assays. Supernatants from stimulated cultures were assayed for growth-promoting activity on the IL-2 and IL-4 responsive cell line CTL.L. In addition, the intensity of Ia Ag expression on B cells present in the anti-CD3-stimulated cultures was monitored by flow cytometry. A typical experiment is shown in Figure 1. As shown in Figure 1*A*, spleen cells cultured in the presence of rIL-4 expressed increased levels of Ia determinants; Figure 1*B* indicates that rIL-4 induce a strong pr liferative response of the CTL.L line used. Although supernatants of spleen cells stimulated with anti-CD3 mAb did not contain growth-promoting activity for the CTL.L line (Fig. 1*D*), B cells present in these cultures displayed a strong increase in Ia expression (Fig. 1*C*). This phenomenon was mediated by IL-4, as the anti-IL-4 mAb 11B11 could abrogate the anti-CD3-induced hyper-Ia expression (Fig. 2). As a control of specificity, LPS-induced Ia expression was not affected by the addition of 11B11 antibodies (Fig. 2). Anti-CD3 in these cultures was insolubilized by plastic adherence, so that the inhibitory effect of 11B11 cannot be due to competition for FcR in culture. In addition, no effect of normal rat Ig was observed in these cultures (data not shown).

*IL-4 secretion requires TCR cross-linking.* Optimal activation by anti-CD3 antibodies requires TCR cross-linking. Multivalent forms of anti-CD3 mAb can be obtained by insolubilization on a solid phase or by binding to an FcR bearing cell. This is seen in the Figure 3, in which different forms of the 145-2C11 mAb have been used to activate naive spleen cells to proliferation (Fig 3*A*) and IL-4 production (Fig. 3*B*). Antibodies were either added in soluble form to cultures, or insolubilized on

[3] Abbreviation used in this paper: CsA, cyclosporin A.

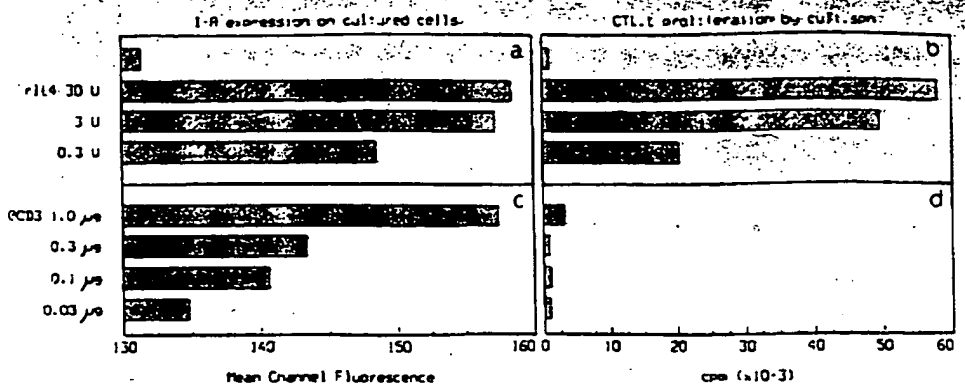


*Figure 1.* Anti-CD3 mediated activation of naive spleen cells: comparison of two different IL-4 assays. Spleen cells were stimulated in vitro by soluble anti-CD3 mAb. After 18 h, cultures were harvested and IL-4 production was monitored by two different methods (see *Materials and Methods* for details). Cultured spleen cells were analyzed for Ia expression (*c*) and culture supernatants assayed for lymphokine content using the CTL.L indicator cell line (*d*). As positive controls, the effect of rIL-4 on the expression of Ia by B cells is shown in *a*, whereas the ability of rIL-4 to promote CTL.L proliferation is presented in *b*. Results are expressed as the mean channel fluorescence of the FITC-positive peak (*a* and *c*) or cpm of $^3$H-thymidine incorporated by the CTL.L cell line (*b* and *d*).

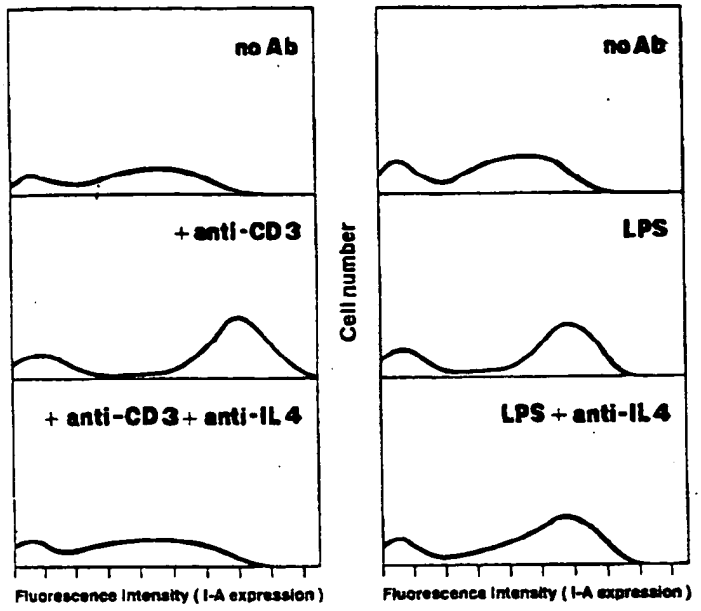


*Figure 2.* In vitro inhibition of increased Ia expression induced by anti-CD3 or LPS by the 11B11 anti-IL-4 mAb. Naive spleen cells were cultured for 18 h in the presence of the indicated antibodies or mitogens before the expression of Ia determinants analyzed by flow cytometry. Final concentrations of reagents were: LPS, 6 μg/ml; anti-IL-4, 25 μg/ml; anti-CD3, 0.1 μg/ml.

plastic as described in *Materials and Methods*. The data show that in soluble form, only intact IgG are able to induce proliferation as well as IL-4 production in vitro. Ins lubilization of all forms of intact or enzyme digested antibodies was effective in mediating activation, showing that the failure of F(ab')$_2$ or Fab fragments to activate T cells in the soluble form was due to insufficient cross-linking. These results indicate that IL-4 production requires T cell activation, because, as demonstrated for IL-2, only conditions that induce T cell proliferation yield IL-4 production. In addition, these experiments further rule out the possibility that endotoxins are responsible for the increased Ia expression, because fragments of the anti-CD3 mAb are only active when cross-linked.

*Only CD4$^+$CD8$^-$ cells secrete IL-4 after anti-CD3 stimulation.* The ability to induce naive spleen cells to secrete lymphokines without long term culture enabled us to phenotype the cells precommited to IL-4 production by depleting from the responding T cell population CD4$^+$ or CD8$^+$ cells. Spleen cells were first treated with anti-CD4 or anti-CD8 mAb and C′ as described in *Materials and Methods*; cells were then stimulated in culture in the presence of the relevant anti-CD4 or anti-CD8 mAb to functionally block contaminating cells that might have escaped C′-mediated lysis. As seen in Figure 4, depletion of CD4$^+$ cells abrogates the increase in Ia expression whereas elimination and blocking of CD8$^+$ cells was without effect. This confirms the results obtained with long term cultured cell lines, which indicate that IL-4 pr duction is restricted to CD4$^+$ T cell lines and clones. Again, it is worth noting that in contrast to IL-4 production, LPS-induced Ia increased expression can occur in the absence of T cells (data not shown).

*Anti-CD3 mAb induce IL-4 synthesis in vivo.* To study the effect of anti-CD3 stimulation in vivo, BALB/c and nude mice received i.v. injections with 25 μg of affinity purified anti-CD3 mAb. In vivo administration of anti-CD3 mAb results in an increase of Ia expression by splenic cells (compare Fig. 5 *e* and *g* to *a* and *c*). As for the in vitro experiments, these cells were identified as B cells by two-color immunofluorescence using IgM-surface staining (data not shown). This effect requires the presence of mature T cells, because class II expression is not

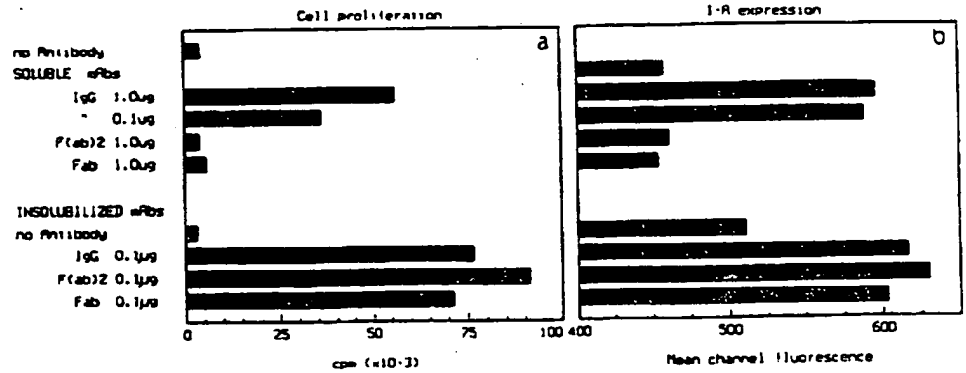


*Figure 3.* Induction of T cell activation by anti-CD3 antibodies or its proteolytic fragments. Anti-CD3 antibodies were added to culture of naive spleen cells in their soluble form or insolubilized on anti-Ig coated plates. Cell proliferation was tested by adding $^3$H-thymidine at the end of the 3 days culture period (*a*). Production of IL-4 was evaluated by the intensity of Ia expression after 18 h of culture (*b*).

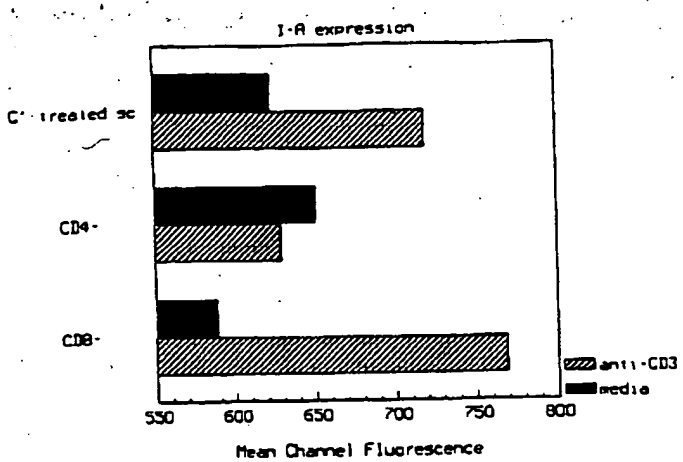


*Figure 4.* IL-4 production by T cell subsets. After C'-mediated depletion, spleen cells were cultured in the presence of subset-specific mAb (20% culture supernatants of respectively GK1.5 for the $CD4^+$ and 83-12-5 for the $CD8^+$ subpopulations). Cells were then cultured in the presence of mitogenic doses of anti-CD3 mAb (0.2 μg/ml) and Ia expression assayed 18 h later.

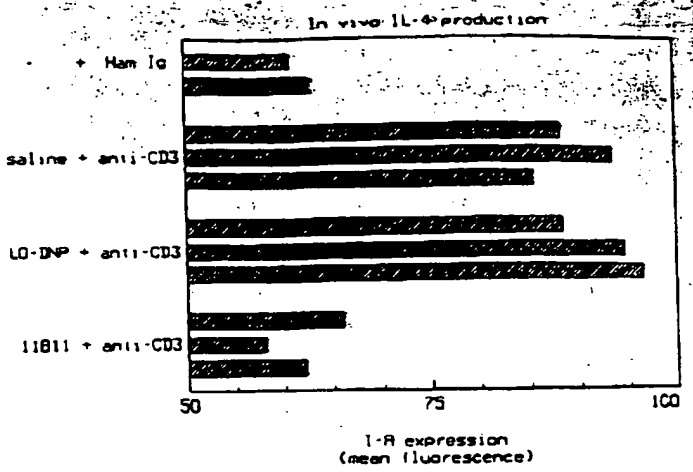


*Figure 6.* Inhibition of anti-CD3 induced hyper-Ia expression in vivo by anti-IL-4 mAb. Mice were pretreated with saline, anti-DNP, or anti-IL-4 rat mAb as indicated in *Materials and Methods* before the i.v. administration of anti-CD3 mAb. Expression of Ia determinants were analyzed by flow cytometry and the results are expressed as indicated in the legend of Figure 1.

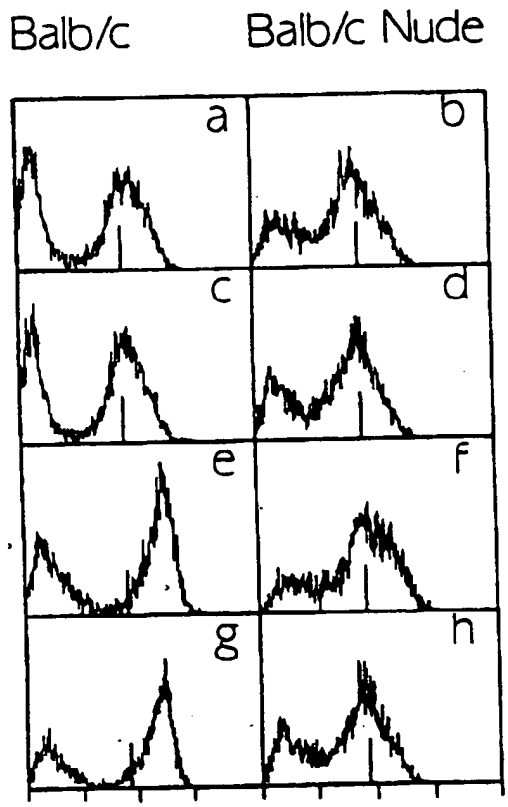


*Figure 5.* Increased Ia expression after in vivo anti-CD3 administration. Naive adult BALB/c (*a*, *c*, *e*, and *g*) or BALB/c nude mice (*b*, *d*, *f*, and *h*) were injected with saline (*a* to *d*) or with anti-CD3 mAb (*e* to *h*). Then 18 h after treatment spleen cells were collected, stained with anti-Ia antibodies, and analyzed by flow cytometry as described. Each histogram represents the fluorescence analysis of an individual mouse.

upregulated in nude mice treated with anti-CD3 (Fig. 5 *f* and *h* compared to *b* and *d*). Increased Ia expression was mediated by IL-4 as suggested by blocking experiments using an anti-IL-4 mAb. Mice were pretreated with saline or with rat IgG1 Ig from either the LO-DNP-2 clone (specific for the hapten DNP) or the 11B11 cell line (specific for the murine IL-4). As shown in Figure 6, pretreatment of mice with the anti-IL-4 mAb but not with the control antibody prevents the anti-CD3-induced hyper Ia expres-

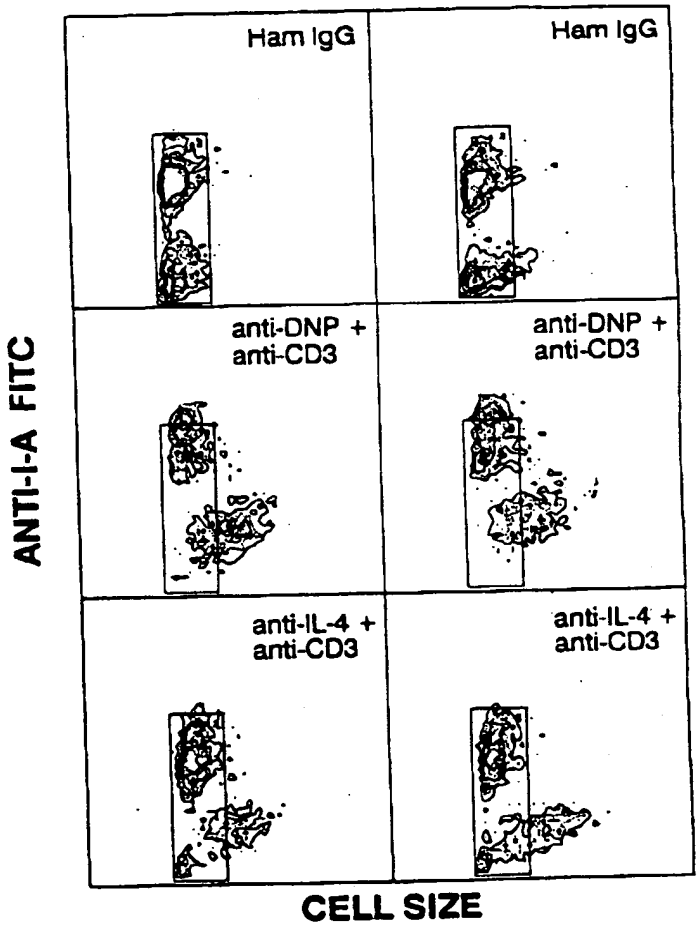


*Figure 7.* Specificity of the anti-IL-4 mAb inhibition: in vivo administration of anti-IL-4 mAb inhibits increased Ia expression but not blastogenesis after anti-CD3 stimulation. The experiment (mice treatment and fluorescence analysis) was performed as described in the legend of Figure 6. Fluorescence data are displayed as a contour plot in which cell size (as measured by forward light scatter in a linear scale on the x axis) is plotted against the log green fluorescence (y axis). Each diagram represents data from an individual mouse.

si n. In Figure 7 the results of an independent experiment are depicted as contour plots comparing cell size and expression of Ia in the spleen of treated animals. The results indicate that in addition to increased expression of class II determinants, anti-CD3 antibodies provoke an increase in cell size by the Ia⁻ population (identified as Thy-1⁺ in other experiments not shown here). It is of note that anti-IL-4 pretreatment only inhibits the increase in Ia expression but does not alter T cell blastogenesis. These observations suggest that the anti-IL-4 antibodies do not interfere with the ability of anti-CD3 mAb to activate T cells in vivo but prevent IL-4 to activate B cells.

Finally, IL-4 production was unequivocally identified by the analysis of RNA isolated from spleen cells after anti-CD3 administration. Total RNA was extracted from control or 145-2C11 injected animals and analyzed by gel lectrophoresis and Northern blotting with an IL-4-specific probe. IL-4 mRNA of 0.6 kb was detected only in anti-CD3 treated animals as seen in Figure 8.

*Inhibition of IL-4 production by CsA.* In vivo experiments suggest that IL-4 secretion by short term activated spleen cells results from induction of gene transcription rather than release of prestored IL-4. This conclusion is further substantiated by the finding that IL-4 production can be blocked by pretreatment with CsA, a fungal metabolite with immunosupressive activity widely used in transplantation medicine (30). The inhibitory effect of CsA on the increase of Ia expression in vitro is shown in Figure 9a. As shown earlier, the increased Ia Ag expression observed in cultures stimulated by anti-CD3 mAb, is blocked by addition of 11B11 mAb. CsA at 1 μg/ml significantly impairs the production of IL-4 in the culture, as judged by the lack of increased Ia expression. CsA does not interfere with the ability of B cells to respond to IL-4, as shown by the fact that when both rIL-4 and anti-CD3 are added to the cultures, no effect of CsA on Ia expression is observed. To further document this finding, the effect of CsA on T cell-depleted cultures stimulated by IL-4 was also tested. As shown in the Figure 9b, anti-CD3 failed to induce IL-4 production in these T cell-depleted cultures, whereas rIL-4 was able to induce an increase in Ia expression. This effect was not sensitive to CsA, indicating therefore that CsA only acts by inhibiting T cell mediated responses. Taken together, these data show that CsA is able to block the induction of IL-4 synthesis by T lymphocytes, in agreement with its previously demonstrated ability to inhibit IL-2 synthesis. Similarly, in vivo administration of CsA prevents increased Ia expression induced by anti-CD3 mAb. For these experiments, mice were treated with 25 mg/kg of CsA 18 and 3 h before anti-CD3 administration. As seen in Figure 10, CsA significantly impairs the anti-CD3-induced hyper Ia expression.

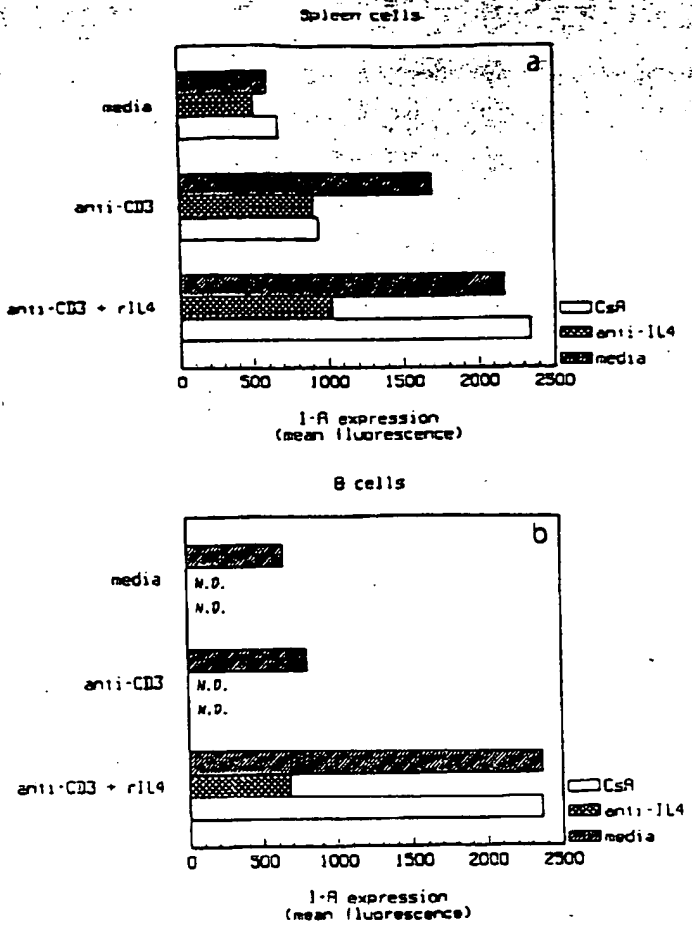


*Figure 9.* CsA inhibits anti-CD3-induced IL-4 production in vitro but does not interfere with the ability of rIL-4 to increase B cell Ia expression. Untreated (*A*) or Thy 1-depleted (*B*) spleen cells were cultured for 18 h in the presence of the indicated stimuli and inhibitors at the following final concentrations: anti-CD3: 0.1 μg/ml; rIL-4: 500 U/ml; 11B11: 50 μg/ml; CsA: 1.0 μg/ml. After culture, cells were harvested and analyzed by dual parameter flow cytometry for surface IgM and Ia expression. Data are expressed as mean channel fluorescence of the surface IgM⁺ Ia⁺ subpopulation (corresponding to B cells).

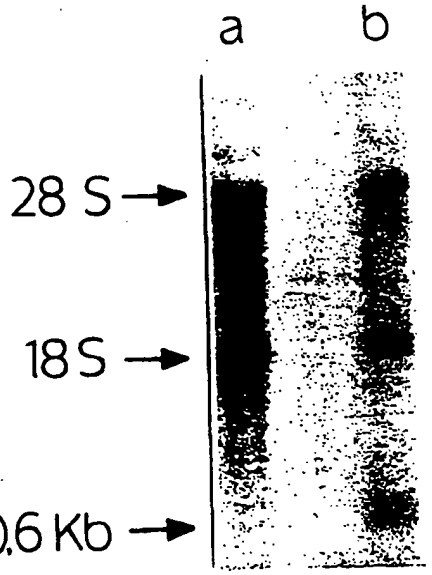


*Figure 8.* Induction of IL-4 mRNA in naive animals. DBA/2 adult mice were injected i.v. with saline (*a*) or 25 μg of anti-CD3 mAb (*b*). Spleen cells were harvested 2.5 h after treatment and total RNA was extracted as described. Five μg of RNA was electrophoresed on agarose gel, transferred to nylon membranes and probed with $p^{32}$ labeled IL-4 mRNA.

## DISCUSSION

The major observation from this study is that IL-4 can be readily secreted by unprimed CD4⁺ CD8⁻ cells after TCR complex cross-linking, both in vitro and in vivo.

The production of IL-4 was detected by the increased expression of class II determinants on B cells present in anti-CD3 activated cultures. This assay proved to be more sensitive than det ction of s luble IL-4 in supernatants.

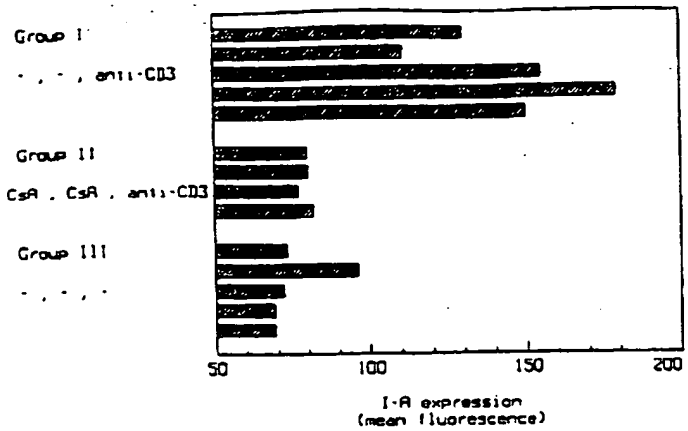


*Figure 10.* CsA inhibits the increased Ia expression induced in vivo by administration of anti-CD3 mAb. Mice were injected with anti-CD3 mAb as described. Group I and group II mice were injected with anti-CD3 mAb. group III animals were left untreated. Group II animals were pretreated with two injections of 35 mg/kg CsA as described in *Materials and Methods*. Data are shown for individual mice in each group and are expressed as the mean channel fluorescence for Ia staining.

a phenomenon consistent with the idea that IL-4 produced in the culture is consumed by resident cells and is no longer available in the supernatant for detection. It is of note that a similar situation was described by Mizuochi et al. (32). who showed that IL-2 production by allostimulated $CD4^-$ $CD8^+$ cells can only be detected if IL-2 uptake by cells in the culture is prevented by the addition of anti-IL-2R antibodies.

Anti-CD3-mediated activation in vitro induces $CD4^+$ T cells to produce IL-4, which in turn activates B cells to higher Ia expression.

This conclusion is supported by the following observations. 1) Anti-IL-4 mAb significantly impair the hyper-Ia expression, induced by anti-CD3 mAb, indicating that IL-4 is produced in vitro and is responsible for the observed B cell activation. 2) IL-4 producing T cells belong to the $CD4^+$ $CD8^-$ subset. The possibility that endotoxins rather than IL-4 were responsible for the increased Ia expression has been carefully ruled out because 1) anti-IL-4 antibodies do not inhibit LPS-induced Ia increased expression and 2) high Ia expression requires insolubilization of anti-CD3 antibodies and the presence of $CD4^+$ cells.

The rapid production of IL-4 in vivo after anti-CD3 administration represents a novel finding. The data presented show that anti-CD3 antibodies activate T cells to IL-4 mRNA transcription and probably IL-4 secretion in vivo, which in turn induce high expression of Ia determinants by B cells. Northern blot experiments show that mRNA specific for IL-4 can be detected as early as 3 h after anti-CD3 administration. Lack of hyper-Ia expression in nude mice indicate that anti-CD3 antibodies must interact with mature T cells to induce IL-4. Finally, inhibition experiments performed in vivo with the 11B11 anti-IL-4 mAb indicate that the lymphokine responsible for the hyper Ia expression is IL-4. The experiments show that inhibition by the 11B11 mAb is specific, because it only blocks B cell activation but does not interfere with anti-CD3 induced T cell blastogenesis.

These observations uggest that activati n of naive T cells to IL-4 production does n t require previous stimulati n and therefore raise questions concerning the role and function of Th subsets in mice. It is of not again that Th subsets in mice are best defined by the mutually exclusive production of lymphokine. We and others have studied lymphokine release from total spleen cell populations, and did not attempt to identify lymphokine producers at the clonal level. However. for the sak of discussi n, we will assume that cells endowed with the ability to produce IL-4 belong to the Th2 subset.

Although $CD4^+$, IL-2-producing cells can be readily identified in polyclonally or allostimulated cultures, many authors (12, 13) have reported that IL-4 could only be detected in supernatants of primed and restimulated cultures. In contrast, our observations indicate that cells able to synthesize IL-4 can be found in the preimmune lymphocyte pool both in vivo and in vitro, provided a sensitive assay is used. Our data are in agreem nt with recent observations reported by several groups. Hayakawa and Hardy (33) have used 2 mAb defining new c ll surface Ag to identify four distinct subpopulations f murine $CD4^+$ cells. Two of these populations show mutually exclusive production of lymphokines, Fraction I secreting IL-2 and Fraction III IL-4 after Con A stimulation. Interestingly, the authors report that only IL-2 and not IL-4 is detectable in the supernatant of unfractionated $CD4^+$ lectin-stimulated cells. Others, using in situ hybridization, have demonstrated that approximately 1 of 300 to 400 spleen cells expresses IL-4 mRNA 24 h after stimulation by Con A (34). These observations and ours challenge the hypothesis recently proposed by Swain and colleagues (13) who observed that IL-4 secreting cells (thus assumed to be the equivalent of Th2 cells) could only be detected after priming. They sugge t that the inability to detect Th2 cells in the preimmune pool is not just a reflection of their low frequency but implies that Th2 cells require Th1-derived factors (such as IL-2) before they can mature into functionally active, lymphokine-secreting cells. Our results would rather suggest that at least some Th2 cells can be found in the preimmune population, and therefore raise the possibility that higher amounts of IL-4 produced in secondary stimulations may result from expansion of a preexisting pool of Th2 cells. This would imply that in contrast to IL-2-producing cells, Th2 cells undergo clonal expansion after Ag encounter. This possibility also suggests that Th2 cells can be stimulated during a primary response and thus play a role in the early phases of B cell activati n, especially because it has been shown that IL-4 can act early in the response of resting B cells (35, 36). It has been shown for example that addition of IL-4 to LPS-stimulated cultures induces uncommitted B lymphocytes to switch to $IgG_1$ and IgE secretion (37).

However, before a definitive conclusion can be reached detection of IL-4-producing cells should be attempted ir Ag-free animals, rigorously raised in pathogen-free con ditions under irradiated diet. Before these experiment: can be performed, we cannot exclude the possibility tha IL-4 is produced by T cells primed by environmental Ag Data recently presented by Lewis et al. (38). who wer able to identify IL-4-secreting $CD4^+$ cell by in situ hybrid ization of human PBL polyclonally activated by $Ca^{2+}$ ic nophore and PMA support this hypothesis. The author have shown that most IL-4-producing T cells belong t the $CD4^+$ $CD45R^-$ subset. which is thought to be enriche in memory T cells.

Alternatively, these cells may belong to the pool of internally activated, large T cells that exist also in Ag-free animals. These cells may represent regulatory cells specific for self determinants (MHC products or Ag receptors on B and possibly T cells) as suggested by Pereira et al. (39).

As anti-CD3 mAb activates all T cells in an Ag-independent manner, it is of no surprise that production of several lymphokines is induced. Hirsch et al. (40) recently showed that mice receiving injections with anti-CD3 mAb displayed IL-3 activity in their serum and some of us have shown that IL-2, IFN-γ, and TNF-α could be detected in the circulation after the first injection of the anti-human CD3 mAb OKT3 in kidney transplant recipients (41). In this report we extend these observations and show that IL-4 is also produced after anti-CD3 administration. This release of lymphokines may explain some of the early side effects associated with OKT3 administration in humans and therefore deserves some attention (41, 42). These considerations prompted us to evaluate the effect of CsA on the production of IL-4 induced by the anti-CD3 mAb. Our observations indicate that CsA is able to block IL-4 production both in vitro and in vivo, extending thus to bulk populations data generated with a T cell clone (34).

Lymphokine release in the periphery may have multiple effects on the host immune system. In particular, we demonstrate here that anti-CD3 administration in vivo, thought to be exquisitively specific for the T cell compartment, affects the B cell subset through IL-4 production. B cells that express higher levels of Ia have been demonstrated to be more efficient APC (43), to be more responsive to T cell-derived help (44), and also to form better aggregates with T cells (45). These modifications could represent an important cofactor in the generation of anti-OKT3 antibodies often observed in the serum of patients undergoing OKT3 therapy (46).

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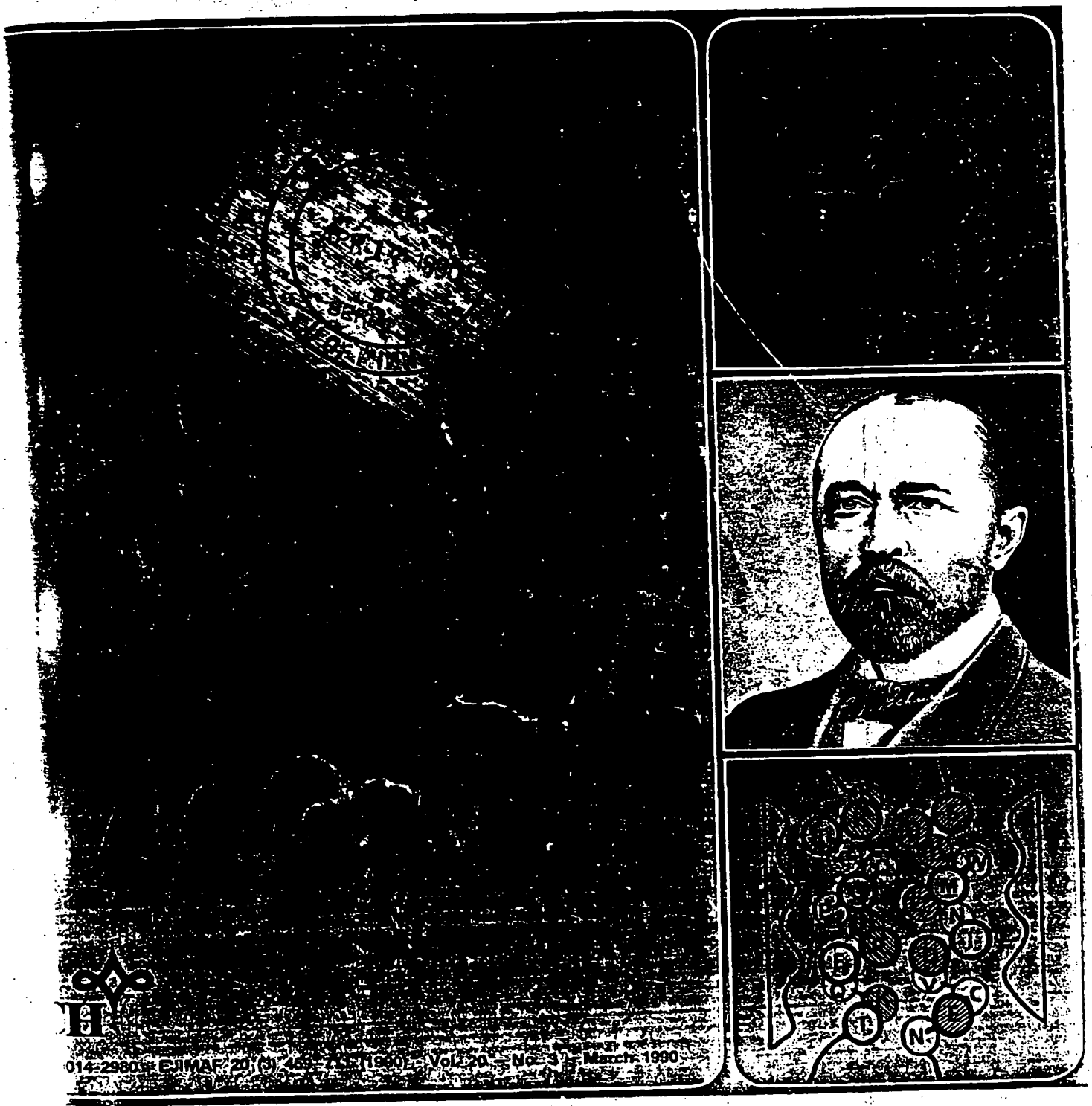

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# Cytokine-related syndrome following injection of anti-CD3 monoclonal antibody: further evidence for transient *in vivo* T cell activation

*In vivo* injection of the hamster anti-murine CD3 monoclonal antibody 145 2C11 into BALB/c mice induces a massive systemic release of several cytokines. Very high circulating levels of tumor necrosis factor are detected both by enzyme-linked immunosorbent assay and L-929 bioassay 90 min following a single injection of 10 μg/mouse 145 2C11. Peak circulating levels of exclusively T cell-derived products such as interferon-γ, interleukin 2 and interleukin 3 are also detected 90 min to 8 h post-injection. Importantly, this cytokine release is transient since none of these cytokines are still present 12 to 24 h post-injection.

In parallel to cytokine release, 145 2C11-treated mice (10 μg/mouse) exhibit somnolence, hypomotility (quantified by actimetry), hypothermia, diarrhea and piloerection. At this dosage, the physical reaction is not lethal and reverses in all mice by 48 h post-injection. Severe but again reversible anatomopathological changes are also observed: massive cellular depletion, necrosis and edema of lymphoid organs, leakage syndrome and inflammatory cell infiltrates of the lung, cell vacuolization, necrosis and vascular congestion of the liver.

All these data are similar to the clinical and immunological manifestations of the OKT3-induced reaction in patients and, thus, provide an invaluable experimental tool to study its mechanisms and explore its prevention.

## 1 Introduction

The hamster mAb 145 2C11 that specifically recognizes the ε chain of the murine CD3 molecule is a potent immunosuppressant. Thus, when administered *in vivo* it significantly prolongs skin allograft survival [1–2]. The antibody exerts its immunosuppressive effect by inducing, in a dose-dependent manner, both depletion of $CD3^+$ cells and antigenic modulation of the CD3/TcR (Ti) molecular complex without physical disappearance of the cells [3–4].

When injected in normal adult mice, 145 2C11 induces an impressive first-dose-related acute sickness whose most characteristic signs are somnolence, hyporeactivity, diarrhea and massive piloerection. This reaction, which is lethal within a few hours when high antibody doses are used (> 100 μg/mice), is highly reminiscent of the complex clinical syndrome observed in human allograft recipients receiving the anti-CD3 mAb OKT3. This latter reaction that essentially includes high fever, chills, headaches and gastrointestinal symptoms is only observed after the first OKT3 injections. Although this systemic syndrome is spontaneously reversible it represents one major side effect associated to the clinical use of OKT3 [5–7]. We have recently shown that in patients this reaction correlates with massive, although transient, release in the circulation of various cytokines including TNF and exclusively T cell-derived products like IFN-γ and IL 2 [8–10].

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In this report the physical and biological characteristics of the murine reaction induced by 145 2C11 have been dissected to give a better delineation of its role and its physiopathology.

## 2 Materials and methods

### 2.1 Mice

Five-to seven week-old BALB/c, DBA/2 and nu/nu Swiss mice were from Iffa Credo (Les Ullis, France). C3H/HeJ were from the Centre National de la Recherche Scientifique (CNRS) animal facilities (Orléans, France).

### 2.2 mAb and treatment schedules

145 2C11 is a hamster IgG mAb [1] specific for the murine CD3 ε chain. GK1.5 is a rat (anti-mouse CD4) mAb initially characterized by Dialynas et al. [11]. mAb-containing ascitis were produced in irradiated (250 rad) DBA/2 mice for 145 2C11 and in nude mice for GK1.5. Ascites were purified by means of 50% ammonium sulfate saturation and DEAE ion-exchange chromatography. BALB/c mice were injected i.v. with a single 10-μg dose of 145 2C11 or GK1.5 diluted in saline (100 μl total injected volume). Mice only injected with 100 μl saline were studied as controls in actimetric studies.

### 2.3 Evaluation of motor activity

The motor capacity of each single mouse was measured by means of an actimetric device. This instrument is a photo cell counter initially described by Dews et al. [12, 13] widely used to study pharmacological modulation of either

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**Abbreviations:** **CM:** Culture medium **rMu:** Recombinant murine **VSV:** Vesicular stomatitis virus **HCSA:** Histamine-producing cell-stimulating activity **GM-CSF:** Granulocyte/MΦ-CSF



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motor capacity or behavioral attitudes [13–15]. Each actimetric device includes six translucent (plexiglass) boxes (26 × 21 × 10 cm). One centimeter above each box support, two photoelectric cells are mounted at a 90° angle. Thus, each tim the mouse crosses one of the two light beams an interruption is recorded by the photo cell. M tor activity of each mouse may then be quantitated as the sum of the recorded photo cell light interruptions in a predefined tim interval.

In all experiments, actimetric measurements were performed during the first 30 min after placing mice within the boxes. This time corresponds to a first phase of "recognition" of the surrounding medium and well reflects the overall receptive and reactive capacity of the animals. Actimetry was performed before and at 4, 24 and 48 h following the injection of each tested mAb. In all experiments mice were marked so that: (a) at each time point a given mouse was introduced in a different box to avoid habituation to surrounding environment and, (b) longitudinal analysis of each single animal could be performed.

### 2.4 Body temperature and diarrhea

In all experiments, mice were kept at a 22 °C to 24 °C room temperature. Rectal body temperature was serially recorded before each actimetric study by means of an electronic heat-sensitive microthermometer (Thermalet, TH8; Bailey Instruments). Body temperature was measured 30 s after introducing the rectal probe for approximately 1 cm. This procedure was repeated before and at 4, 24, 48 and 72 h following the injection. Diarrhea was scored as present or absent at the same time points detailed for body temperature.

### 2.5 Cytokine assays

Blood samples were drawn before and at 90 min, 4, 8 and 24 h following the mAb injection. Blood was collected at 4 °C in aseptic tubes devoided of additive and LPS free (as assessed by limulus assay). After clotting, sera were immediately recovered by centrifugation at 4 °C and aliquots were stored at −80 °C until tested for cytokine activities. All mouse groups were monitored longitudinally, sera being pooled from the same mice after collection at the various time points.

### 2.6 TNF

TNF was measured in parallel by means of a bioassay using L-929 mouse fibroblasts and a specific ELISA. TNF bioassay was performed as described elsewhere [16, 17]. Recombinant murine TNF (rMuTNF) was used to set up the standard curve [16, 17]. Viability was assessed both microscopically and by vital dye uptake. The technique used for TNF ELISA has already been reported in detail [18]. Briefly, ELISA microplates were precoated with the TNF-specific hamster mAb TN3-19.12. One hundred-microliter serial dilutions of test samples were added for 1 h at 23 °C and the binding revealed by means of a rabbit anti-rMu TNF serum. rMu TNF was used as a standard.

### 2.7 IL 1

The proliferation of the human astrocytoma U-373 cell line was used to test IL 1 activity according to the method described by Lachman et al. [19]. rMu IL 1 was used to set up the reference standard curve. Circulating IL 1 was assessed in our model in both mouse sera and plasma [(plasma was drawn in EDTA-supplemented tubes (LPS free)] after a double chloroform extraction [20]. In our hands neither TNF nor IL 2 had any proliferative effect on U-373 cells.

### 2.8 IFN

IFN was assessed by measuring the inhibition of the cytopathic effect exerted by the vesicular stomatitis virus (VSV) on Res.L-929 cells that are a particular sublin of L-929 cells resistant to the cytotoxic effect of TNF. The test was performed as described in detail elsewhere [21]. Cell viability was assessed 24 h later, microscopically and by crystal violet staining. Partially purified IFN α/β (kindly given by Dr. I. Gresser, Villejuif, France) was used as a reference preparation. For inhibition experiments, sera were preincubated 1 h at 37 °C and 1 h at 4 °C, at different dilution starting 1/20 with the appropriate concentration of the hamster anti-murine IFN-γ mAb H-22 before assessing the antiviral activity.

### 2.9 IL 2

IL 2 activity was tested on the IL 2-dependent murine cytotoxic T cell line (CTLL-2) as previously described [22]. Reference curves were set up by using both natural and recombinant IL 2. Inhibition tests were performed using two specific anti-Mu IL 2R mAb: 5A2 and PC61. For these experiments serial sera dilutions (starting 1/30) were preincubated 1 h at 37 °C and then 1 h at 4 °C with either the 5A2 or PC61 mAb at the appropriate concentration, before assessing the proliferative capacity on CTLL-2.

### 2.10 IL 3 and granulocyte MΦ CSF (GM-CSF)

IL 3 and GM-CSF activities were assessed by means of the histamine-producing cell-stimulating activity (HCSA) that measures the histamine released by C57BL/6 BM cells in response to the tested sera [23]. Histamine determination is performed by an automated fluorimetric method [24]. In our hands 145 2C11 exhibited minor HCSA. Thus, HCSA in the sera of 145 2C11-treated mice were correct d for this background values. Inhibition tests were performed by preincubating 3 h at 37 °C serum samples with specific polyclonal anti-Mu IL 3 and/or GM-CSF antibodies (anti-Mu IL 3 is a kind gift of Dr. Ziltener, Vancouver; anti-Mu GM-CSF is a kind gift of Dr. Mermod, Biog n).

### 2.11 Histological studies

Mice were killed before and at 24 and 48 h following 145 2C11 injection. The different organs studied were immediately taken, fixed in Dubosq Brazil fixative, embedded in 50% paraffin and 50% paraplat. A standard stain with hematoxilin eosin safran was performed.

### 2.12 Statistical methods

Variations of mouse motor activity with time, according t the treatment administered, was analyzed by an analysis of variance (ANOVA) and repeated measures [25]. *Post-hoc* tests were used to delineate temporal differences between treatment and placebo at each time [26]. The analysis was made using NCSS software [25]. All th other statistical analysis were performed using Student's *t*-test.

## 3 Results

### 3.1 Physical reaction induced by 145 2C11 injection

#### 3.1.1 Clinical assessment

All mice injected with 145 2C11 showed an impressive, alth ugh reversible, physical syndrome associating a wide variety f symptoms as soon as 1 h following the injection. Mice were somnolent, hyporeactive and presented hypothermia, diarrhea and piloerection. At higher 145 2C11 dosages this reaction may be lethal. Single injections of doses of 145 2C11 $> 50$ μg were associated with high mortality (*i.e.* 50% mortality 2–3 days after an injection of 50 μg; 80% mortality at 2 days following one injection of 100 μg; 100% mortality at 2 days following one injection of 400 μg).

#### 3.1.2 Actimetric evaluation

At time 0, before treatment, normal BALB/c mice showed a mean motor activity of 372 ± 19 (mean ± SEM) movements per 30 min (mvts/30'). Statistical analysis using ANOVA with repeated measures showed that mice behavioral activity was significantly decreased after 145 2C11 treatm nt ($p < 0.0001$). *Post-hoc* tests were performed to analyze motor activity at each time point and showed a very significant decrease at 4 h following a 10-μg 145 2C11 inj ction ($p < 0.0001$) (Fig. 1). The minor decrease in motor activity expressed at 4 h in the control group (mice injected with saline) was nonsignificant and due to mouse "habituation" to the surrounding environment. Preliminary experiments (data not shown), using serial measurements at 1, 4, 5, 7, 8, 24 and 48 h following 145 2C11 injection revealed that the reaction was the most intense at 4 h post-injection. Hypomotility of 145 2C11-treated mice was still significant at 24 h post-injection. Mice started to recover at 24 h post-injection and normal motor activity was regularly evidenced 48 h post-injection.

#### 3.1.3 Hypothermia and diarrhea

Rectal temperature was measured before each actimetric study. A significant reduction in body temperature ($p < 0.0001$) was noted in all anti-CD3-treated mice at 4 h post-injection. This hypothermia was still significant at 24 h. Recovery started at 48 h and was complete by 72 h post-injection (Fig. 1). Diarrhea was transiently present in all anti-CD3-treated mice. It was first recorded by 4 h post-injection and had disappeared by 24 h.

### 3.2 Cytokine release in 145 2C11-treated mic

#### 3.2.1 TNF

##### 3.2.1.1 L-929 bioassay

TNF was not detected before 145 2C11 injection in the sera f BALB/c mice. In our hands, the first serum dilution used (1/20) did not provoke nonspecific cytotoxicity on L-929 cells. By 90 min following 145 2C11 injection a very significant increase in circulating TNF was observed in all mice (mean ± SEM: 64 ± 9.8 U/ml). Fig. 2 shows the results obtained with five different representative serum samples, each consisting of a pool of five different mouse sera. Importantly, the kinetics of TNF release was very sharp. No activity could be detected in any serum drawn at 4, 8 and 24 h after the 145 2C11 injection.

##### 3.2.1.2 ELISA

Concordant results were obtained: a peak of TNF was observed in three out of the five pooled sera 90 min post-injection (mean ± SEM: 1.18 ± 0.48 ng/ml). It is important to note that both the bioassay and the ELISA may detect TNF and lymphotoxin.

#### 3.2.2 IL 1

None of the BALB/c sera or plasma tested by means of the U-373 cells bioassay was able to induce proliferation even when a double chloroform extraction was performed.

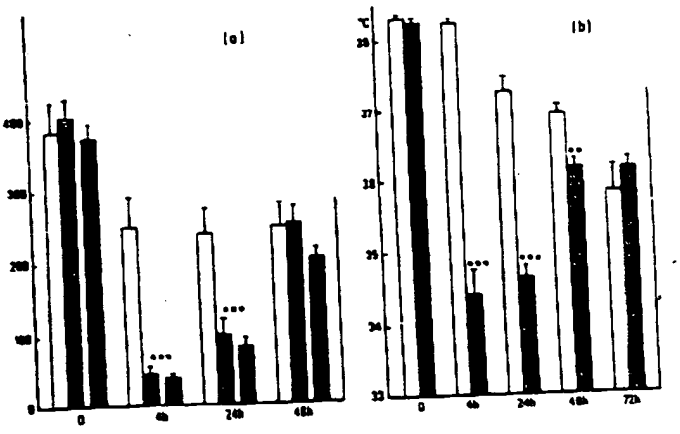


*Figure 1.* Actimetric evaluation (a) and body temperature (b) measurements of BALB/c mice after one single 10-μg 145 2C11 injection (dashed column) compared at each time point with controls treated with saline (open column). Six to eight animals were included in each studied group. For actimetry a mean of six experiments is shown in full columns. Actimetric results are expressed as the number of movements per 30 min. Since mice are not strict homeotherms, hypothermia is the regular response to LPS and/or cytokine injection when the environmental temperature is between 22 °C and 25 °C*. This is at variance with humans in wh m hyperthermia is observed. *** $p < 0.0001$; ** $p = 0.002$.

* Berry, L. J., *Fed. Proc.* 1966. 25: 1264.

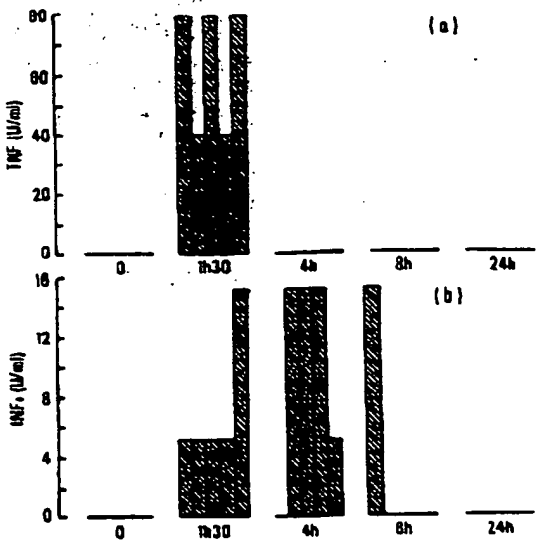


*Figure 2.* Serum TNF (a) and IFN-γ (b) levels in 145 2C11-treated BALB/c mice. Five groups of five mice each were tested longitudinally at each time point. All five groups were positive for TNF in L-929 bioassay 90 min following 145 2C11 injection. Values ranged 40 to 80 U/ml with a mean of 64 ±9 U/ml (mean ± SEM). IFN-γ was detected 90 min to 4 h after the injection, levels ranged 7 ± 2 U/ml (mean ± SEM) at 90 min and 10 ± 3.16 U/ml at 4 h.

### 3.2.3 INF-γ

A subline of L-929 mouse, Res L-929, that is resistant to the cytotoxic effect of TNF was used to detect IFN. Even at the highest concentration used (1/20), none of the sera showed an intrinsic cytotoxic activity on Res. L-929 cells. Before 145 2C11 treatment no detectable anti-viral activity was observed. Following 145 2C11 injection a very significant increase in such activity was evidenced depending on the samples, peak levels (> 15 U/ml) were reached between 90 min and 8 h post-injection (Fig. 2). By 24 h, in all experiments, none of the serum samples showed any residual antiviral activity (Fig. 2). Using the anti-Mu IFN-γ mAb H22, 100% inhibition was noted in all positive samples (data not shown).

### 3.2.4 IL 2

Before 145 2C11 injection, normal BALB/c mice sera did not induce any proliferation of the CTLL-2 cells. 145 2C11 by itself did not either induce any proliferation of the cell line. At 90 min and 4 h following 145 2C11 injection all sera triggered CTLL-2 proliferation (Fig. 3). As with the other cytokine activities tested, the release was transient and sera drawn at 8 h post-injection did not show any residual activity (Fig. 3). Inhibition experiments performed using two different anti-Il 2R mAb (PC61 and 5A2) confirmed that all the detected activity could be abolished by such treatment (data not shown).

### 3.2.5 IL 3

Before treatment none of the sera showed any detectable IL 3-mediated activity. Significant HCSA were detected at 4 and 8 h after 145 2C11 injection. This activity completely disappeared by 24 h post-injection (Fig. 3). Inhibition experiments showed 78% inhibition of the detected HCSA using the anti-IL 3 antisera while no significant inhibitory capacity could be obtained with the anti-GM-CSF antisera (data not shown).

### 3.3 Histopathological abnormalities

Lymphoid organs namely, thymus, spleen and LN as well as non-lymphoid organs namely, liver, lung, digestive tract, heart and kidney were examined. Before 145 2C11 injection, all organs were normal (Figs. 4 and 5). Conversely, 24 h after the injection, severe microscopic modificati ns were noted in lymphoid organs (important cell necrosis and edema), and non-lymphoid organs: lung presented severe alveolar congestion, leakage syndrome and cellular infiltrates; liver showed severe sinusoidal congestion, hepatocyte vacuolization and bouts of focal tissue necrosis (Figs. 4 and 5). Digestive tract presented moderate capillary congestion and submucosal edema. Neither the heart nor the kidney were modified.

### 3.4 Physical reaction and cytokine release in GK 1.5 (anti-CD4)-treated BALB/c mice

To exclude that the effects observed following 145 2C11 injection were related to eventual LPS contamination of the preparation we used as controls BALB/c mice (10 μg/mouse i.v.) treated with another anti-T cell mAb namely GK1.5 isolated from ascites fluid by means of the same purification procedures used for 145 2C11. In all mice, GK1.5 was well tolerated; no physical reaction was observed in any of them.

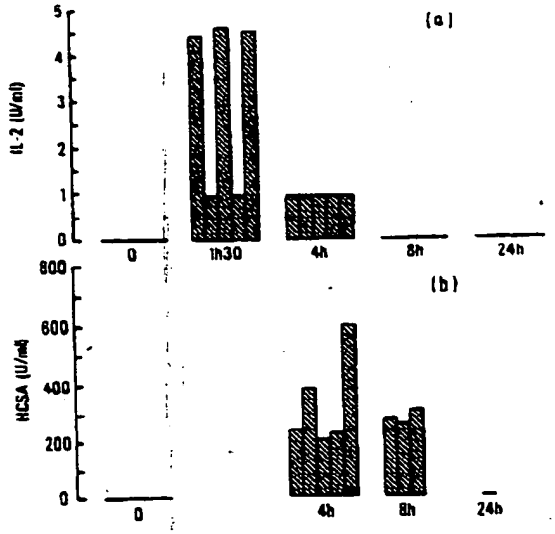


*Figure 3.* Serum IL 2 (A) and IL 3 (B) levels in 145 2C11-treated BALB/c mice. IL 2 (mean ± SEM) values after 145 2C11 injection: 3 ± 0.88 U/ml at 90 min and 0.9 U/ml at 4 h. No IL 2 is detected thereafter. IL 3 (mean ± SEM) values after 145 2C11 injection (HCSA U/ml): 323 ± 74 U/ml at 4 h, 271 ± 15 U/ml at 8 h and 4 U/ml at 24 h (one sample tested representing a pool of five different groups).

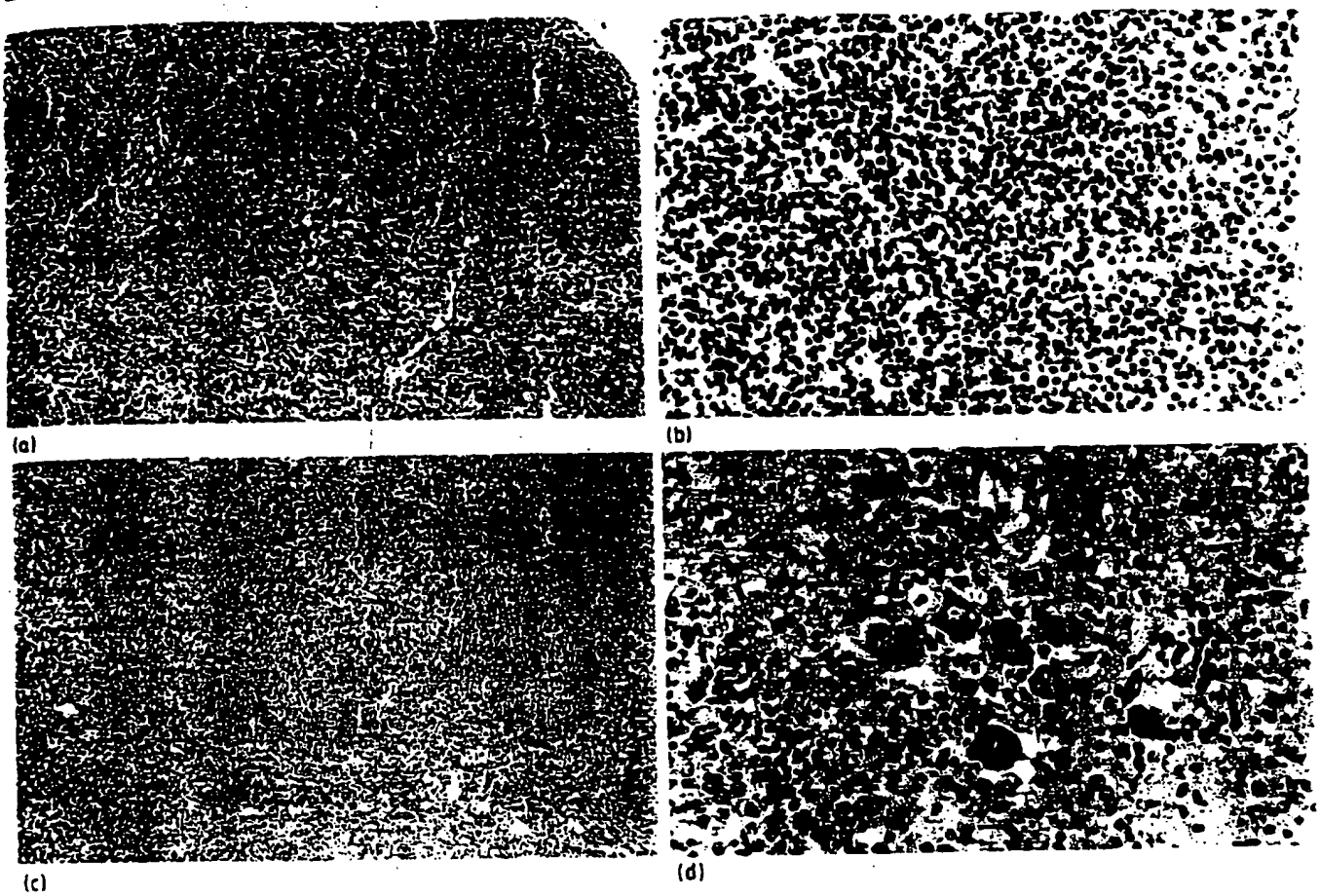


*Figure 4.* Microscopical analysis of lymphoid organs: (a) thymus (× 250) and (c) spleen (× 100) before 145 2C11 treatment are normal, showing normal architecture (arrow). Severe changes are noted in both (b) thymus (× 400) and (d) spleen (× 400) 24 h after the injection, consisting of major cell necrosis, capillary congestion dissociating edema (arrows) and MΦ infiltration in spleen (arrow).

Moreover, none of the various tests described above could evidence any systemic cytokine release following GK1.5 injection (data not shown).

## 4 Discussion

Anti-CD3 mAb are probably among the most potent immunosuppressive agents available and some of them are presently largely used in several transplantation centers either to prevent or treat allograft rejection [4–7]. Contrasting with their profound immunosuppressive potency anti-CD3 mAb express very significant, although transient, *in vivo* activating properties. The present results show that a single 10-μg injection of the anti-mouse CD3 mAb namely, 145 2C11 induces a bulk release of various cytokines in the systemic circultion. Thus, a peak of circulating TNF was detected 90 min after antibody injection by using both a bioassay and a specific ELISA. This TNF release presented sharp kinetics: *i.e.* levels were undetectable by 4 h post-injection.

Importantly, exclusively T cell-deriv d products were detected in the serum of treated mice. Significant antiviral activity was observed at 90 min and 4 h following the injection. Given the described presence of TNF in the sera at 90 min, this antiviral activity was assessed by using a subtype of the L-929 cell line that is resistant to the cytotoxic effect of TNF. Inhibition experiments using a specific anti-IFN-γ mAb demonstrated that this was the cytokine mediating the observed antiviral activity. Similarly, at 90 min and 4 h post-injection, sera from 145 2C11-treated mice induced significant proliferation of CTLL-2 cells that was IL 2 mediated, as assessed in inhibition experiments using specific anti-IL 2R mAb. The presence of circulating IL 3, 90 min to 8 h following 145 2C11 injection was assessed by evaluating HCSA. Since HCSA is known to be mediated by either IL 3 or GM-CSF [23], specific mAb were used to identify the cytokine responsible for this activity in the sera of treated mice.

All these results fully confirm and extend previous data obtained in OKT3-treated renal allograft recipients [8–10]. In such patients, the massive cytokine release gives rise to an acute clinical syndrome characterized by fever, chills, headaches, diarrhea, vomiting and seldom hypotension and respiratory distress [4–7]. We have observed a similar physical reaction in 145 2C11-treated mic . This reaction was quantitated in mice by monitoring body temperature and motor activity (using an actimetric device). Four hours f llowing one single injection 145 2C11-treat d mic exhibited a very significant decrease of the body temperature. An

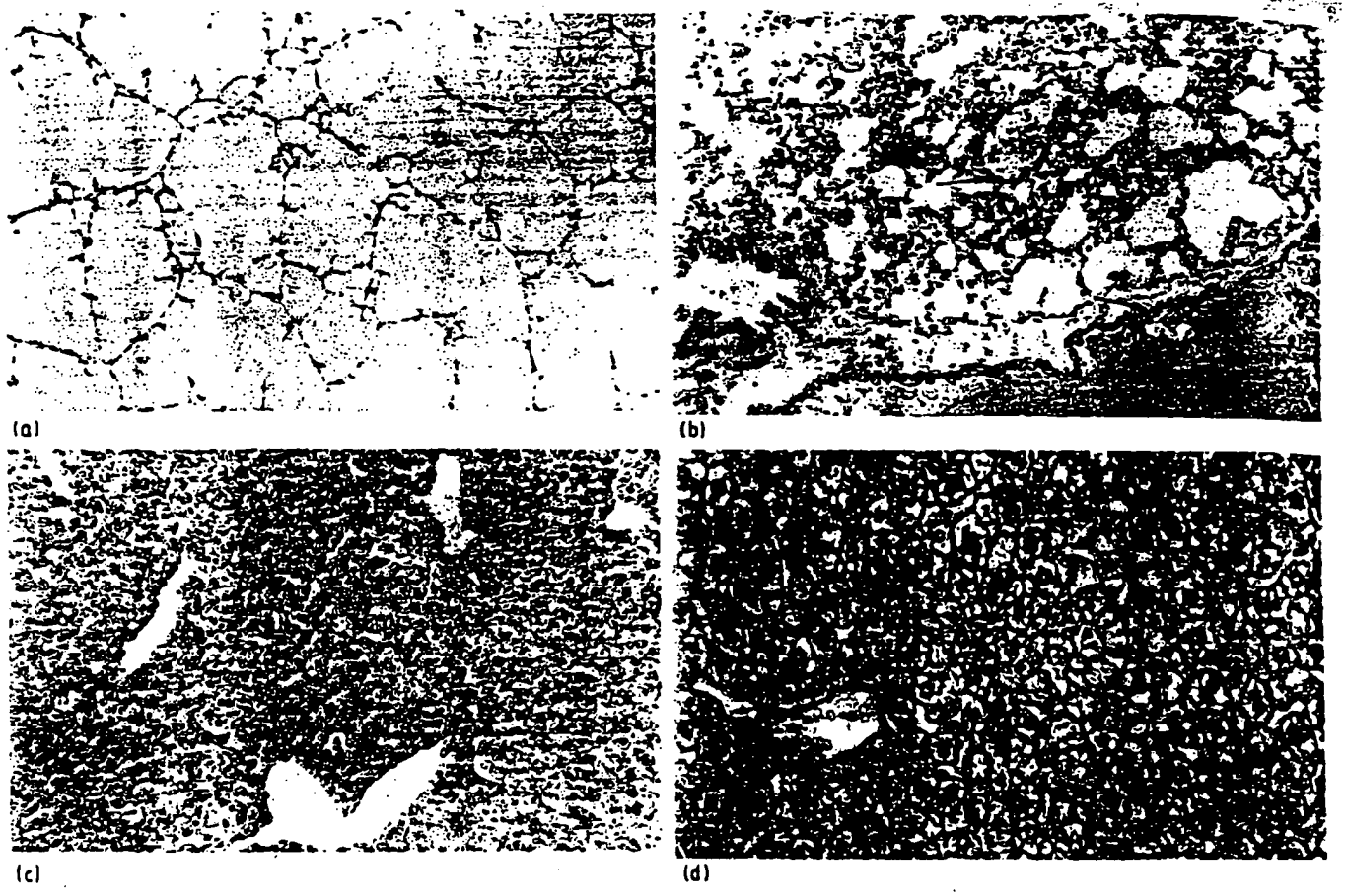


*Figure 5.* Histological analysis of both lung and liver shows major lesions 24 h after 145 2C11 injection. (b) Lung (× 400) presented severe alveolar congestion, cell infiltration with RBC leakage (arrow) and even vascular thrombosis (arrow). (d) Liver (× 400) showed severe sinusoidal congestion, hepatocyte vacuolization and bouts of focal tissue necrosis (arrow). Normal lung and liver tissues are shown in (a) (× 400) and (c) (× 250).

impressively reduced motor capacity was also observed that reflected well the lethargic state of the animals. Importantly, as in OKT3-treated patients, in mice the 145 2C11-induced physical syndrome was spontaneously reversible [4–7]. Elements strongly suggesting a cause-effect relationship between the cytokine release and the systemic clinical syndrome described are: the superimposable kinetics of both phenomena and the similarity between anti-CD3-induced symptoms and the ones reported following *in vivo* injection of recombinant TNF [27], IFN-γ [28] and IL 2 [29]. Moreover, we recently obtained preliminary data in mice showing that injection of mAb specifically directed to the involved cytokines (in adequate amounts and timing) before 145 2C11 administration may prevent the reaction.

The described murine model also demonstrated that the cytokine release induced by one single low dose of anti-CD3 mAb is sufficient to provoke important anatomopathological abnormalities on both lymphoid and non-lymphoid organs. Cellular necrosis and edema were observed in the spleen and LN. Direct cell labeling showed that, at least at these dosages, only partial $CD3^+$ T cell depletion was achieved; the remnant T lymphocytes showing antigenic modulation of CD3/TcR molecular complex (data n t shown). Conversely, in the thymus, while cortical areas disappeared (probably due to the antibody induced stress, *i.e.* endogenous corticosteroids release), non-antigenically modulated T lymphocytes expressing the mature phenotype ($CD3^+CD4^+$ or $CD3^+CD8^+$) were still detected. The hypothesis that this anti-CD3-mediated effect on thymus is more related to systemic massive release of central or peripheral stress hormones than to a direct action of the mAb, is at present being explored.

Concerning non-lymphoid organs, one must underline the major effect of 145 2C11 on both lung and liver which are highly reminiscent of those described in detail by Talmadg et al. [30] following *in vivo* treatment with TNF and/or IFN-γ.

It remains difficult to determine the precise origin of the cytokines produced following 145 2C11 mAb administration. Two main cell source(s) may be envisioned namely, monocyte/MΦ and T cells. Triggering of monocytes/MΦ activation that occur through opsonization and subsequent lysis of anti-CD3-coated Tcells does not seem t represent the only cellular compartment contributing to the cytokine release. In fact, other anti-Tcell (anti-CD4 and anti-CD8) mAb inducing opsonization and/or depletion that have been used *in vivo*, in several experimental models as w ll as in humans, d not provoke the impressive cytokine release

observed with anti-CD3 mAb [31–33], confirming the results obtained using the GK1.5 mAb. Moreover, exclusive monocyte/MΦ activation, as th on induced by *in vivo* injection of LPS leads to systemic release f TNF but n t of T cell-related cytokines [34] nam ly, IFN-γ, IL 2 and IL 3, as found with 145 2C11. Finally TNF, although b ing a major MΦ-derived product, may also be produced by anti-CD3-stimulated T cells [35, 36]. This model provides a unique tool to approach different practical and fundamental issues related to clinical use of anti-CD3 mAb. Notably, they will offer an easy way of testing the effectiveness of various therapeutical regimens (such as: corticosteroids [37, 38], anti-cytokine antibodies, anti-cytokine receptor antibodies, cyclooxygenase inhibitors) in preventing the anti-CD3-induced cytokine release and related physical syndrome. In fact, this reaction is one major pitfall preventing extension of OKT3 treatment to clinical settings other than transplantation (namely, autoimmunity). Additionally, they will give further insight into the physiology of the cytokine cascade and the various synergims [39–47] and feed-back mechanisms that exist between these different molecules.

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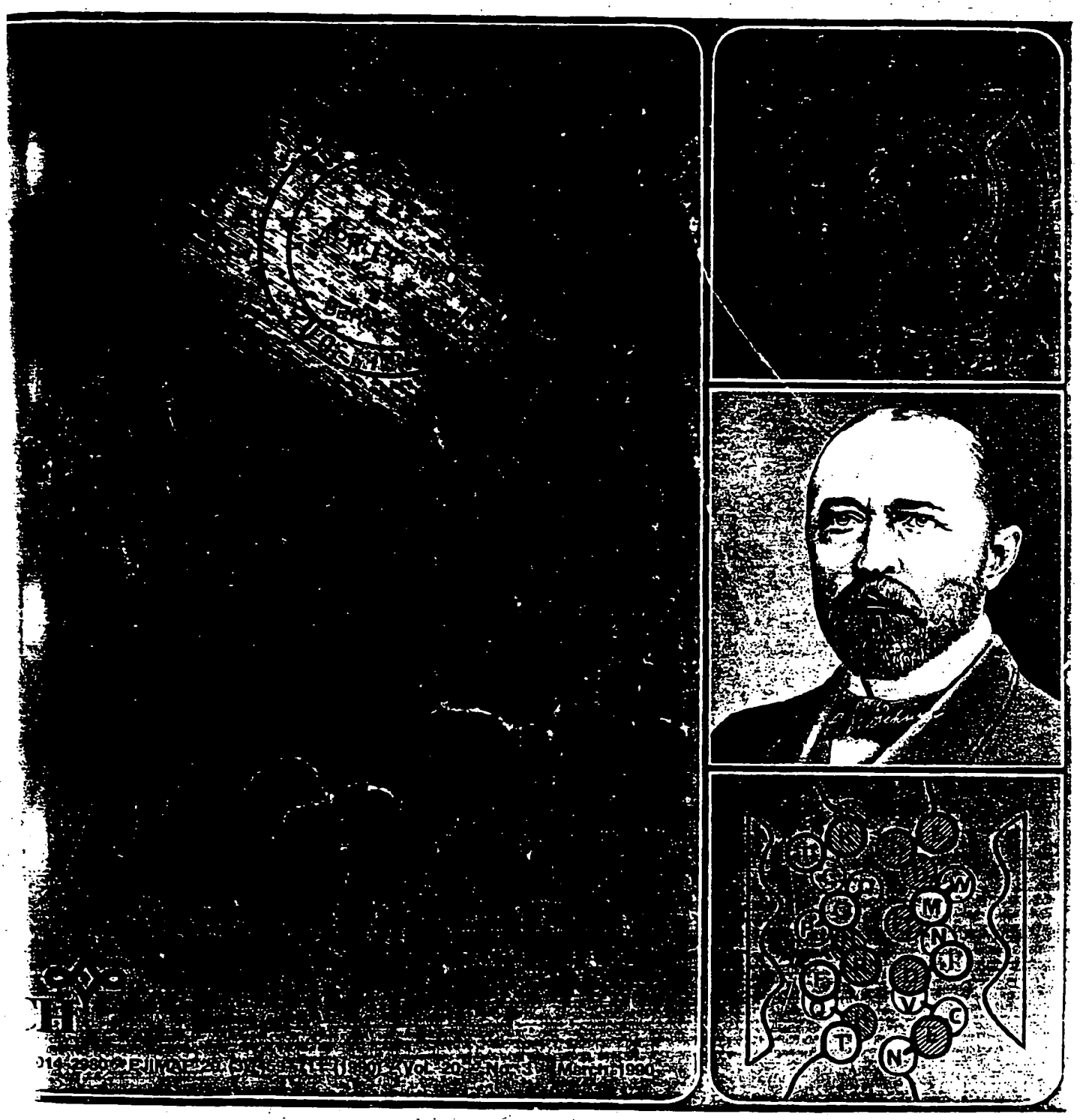
# EUROPEAN JOURNAL OF IMMUNOLOGY 3/1990



Short paper



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# Hypothermia and hypoglycemia induced by anti-CD3 monoclonal antibody in mice: role of tumor necrosis factor*

The possible involvement of tumor necrosis factor-α (TNF) in the metabolic disturbances induced by anti-CD3 monoclonal antibodies (mAb) was analyzed in DBA/2 mice injected with 50 μg of the anti-murine CD3 mAb 145-2C11. First, we found that 145-2C11 induces a profound hypothermia maximal between 3 h and 6 h after the injection (at 3 h: $-3.0 \pm 0.1$ °C) as well as hypoglycemia (blood glucose levels at 6 h and 24 h: $76 \pm 13$ mg/100 ml and $92 \pm 22$ mg/100 ml, respectively, $p < 0.001$ as compared with control values). These metabolic changes are preceded by the release of TNF into the circulation (peak serum TNF levels at 2 h: $50 \pm 23$ pg/ml, $p < 0.01$ as compared with controls). The release of TNF induced by 145-2C11 depends on the effect of the mAb on T cells as it is not observed in athymic nude mice while lipopolysaccharide-resistant C3H/HeJ mice also display a significant rise in serum TNF (peak levels at 2 h: $59 \pm 44$ pg/ml). Pretreatment of DBA/2 mice with 12 mg of rabbit anti-murine TNF antibodies completely prevents the hypothermia while the hypoglycemia is significantly attenuated. Finally, $F(ab')_2$ fragments of 145-2C11 induce only a transient hypoglycemia (blood glucose levels at 6 h: $109 \pm 14$, $p < 0.001$ as compared with controls) but neither hypothermia nor significant TNF release. We conclude that TNF is a major mediator of the acute metabolic changes induced by the intact form of 145-2C11.



## 1 Introduction

mAb directed against the CD3 complex of T lymphocytes represent potent immunosuppressive agents both *in vitro* and *in vivo* [1–4]. Before inhibiting T cell functions, they induce a transient T cell activation resulting in the release of different cytokines [5]. Indeed, we and others recently observed high serum levels of TNF-α, IFN-γ and IL 2 in the hours following the first injection of OKT3 in kidney transplant recipients [6, 7]. Thus, the well-known first-dose reactions commonly experienced by OKT3-treated patients [2, 3] could be mediated by cytokines.

The availability of an anti-mouse CD3 mAb (145-2C11) which shares many properties with OKT3 [8] allows the experimental investigation of the acute metabolic changes induced by this type of mAb. In the present study, we observed that mice injected with the 145-2C11 mAb develop acute hypothermia and hypoglycemia in association with the release of TNF in the circulation. In order to determine the role played by TNF in the pathogenesis of these metabolic changes, we analyzed the effects of the administration of rabbit anti-TNF antibodies prior to the injection of 145-2C11 mAb.

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## 2 Materials and methods

### 2.1 Mice

Six to 9-week-old female mice of DBA/2, *nu/nu* and *nu/+* strains were obtained from Iffa Credo (L'Arbresle, France). LPS resistant C3H/HeJ mice were purchased from Olac (Bicester, GB).

### 2.2 Production and purification of anti-CD3 mAb

The hamster mAb 145-2C11 directed against mouse CD3 [8] was prepared from culture SN of 145-2C11 hybridoma cells by affinity chromatography on protein A-Sepharose (Pharmacia, Uppsala, Sweden).

$F(ab')_2$ fragments of 145-2C11 mAb were prepared by incubating purified mAb with pepsin (Worthington, Freehold, NJ) at a ratio of 100:2 (w:w), in 0.1 M sodium acetate buffer, pH 4.5, for 20 h at 37 °C. The pH of the solution was then adjusted to 7.4 with 1 M Tris-HCl before dialysis against PBS for 24 h. Undigested mAb was removed by adsorption on protein A-Sepharose. The purified $F(ab')_2$ fragments were shown by gel electrophoresis and ELISA using a mAb specific for the Fc portion of the 145-2C11 (clone AH6, generated in our laboratory) to contain < 1% of intact IgG; in addition, they were unable to induce spleen cell proliferation in fluid phase [9]. The immunosuppressive potential of these $F(ab')_2$ fragments was maintained *in vivo* as their injection induces a major depression in cytolytic T cell activities against alloantigens (data not shown).



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### 2.3 Rabbit anti-TNF antibodies

Rabbit anti-murine TNF IgG antibodies ($6 \times 10^3$ neutralizing units/mg) were a kind gift from Dr. E. A. Havell (Trudeau Institute, Saranac Lake, NY). Th IgG fraction of a pool of normal rabbit serum was purified by ion-exchang chromatography and used as control.

### 2.4 Measurement of blood glucose levels

Blood glucose levels were determined using a standard micromethod with Glucostix strips and Glucometer II M (Miles Laboratories, Elkhart, IN).

### 2.5 Determination of TNF serum levels

The serum levels of TNF were determined by a cytotoxicity assay on actinomycin-D-treated WEHI-164 clone 13 cells [10], the number of surviving cells being determined by the use of MTT tetrazolium (M-2128, Sigma Chem. Co., St. Louis, MO) [11]. Briefly, WEHI-164 clone 13 cells were trypsinized and seeded in flat-bottom microtiter plates at a concentration of $3 \times 10^4$/100 µl medium in each well. Twenty-four hours later, serial dilutions of the serum samples were added in the presence of actinomycin D at a final concentration of 1 µg/ml. After 18 h of incubation at 37 °C, 20 µl of MTT (5 mg/ml) were added before a further incubation for 6 h at 37 °C. After the addition of 10% SDS-0.01 M HCl, the plates were incubated overnight and the absorbance at 590 nm was measured by a multiwell scanning spectrophotometer. Results were expressed in pg/ml by reference with the cytotoxic activity of a standard preparation of recombinant murine TNF expressed in *E. coli* [12]. The latter had a specific activity of $7.5 \times 10^7$ U/mg measured on L-929 cells, 1 U corresponding to the amount of murine rTNF able to induce 50% of the maximal cytotoxicity (the same TNF preparation exhibited a 40-fold higher specific biological activity on WEHI-164 clone 13 cells).

### 2.6 Experimental protocol

In a first series of experiments, DBA/2 mice were injected i.v. either with 50 µg of intact 145-2C11 mAb or with an equivalent molar amount (33 µg) of $F(ab')_2$ fragments. As control, a group of mice received an i.v. injection of 50 µg of hamster IgG purified from a pool of normal hamster serum. Rectal temperature was sequentially measured by a digital thermometer during the 24 h after the injection of the different antibody preparations and serial blood samples were obtained by retroorbital puncture for determination of serum TNF levels and blood glucose levels. In order to analyze the role of TNF in the metabolic disturbances induced by intact 145-2C11 mAb, a group of mice received an i.v. injection of 12 mg of anti-TNF antibodies 90 min before the administration of the mAb. Another group of mice was pretreated with the same amount of control rabbit IgG.

### 2.7 Statistical analysis

Comparison between groups were done by the Student's *t*-test for blood glucose and serum TNF levels and Wilcoxon's rank sum test for rectal temperatures.

## 3 Results

### 3.1 Hyp thermia after injection of 145-2C11 mAb

As shown in Fig. 1, intact 145-2C11 induced a profound hypothermia maximal between 3 and 6 h after the injection (mean ± SD at 3 h: $-3.0 \pm 0.1$ °C, $p < 0.001$ as compared with preinjection temperatures). In contrast, variati ns in temperatures after injection of either control hamster IgG or $F(ab')_2$ fragments of 145-2C11 mAb were always < 1 °C (Fig. 1). The hypothermia induced by 145-2C11 mAb was transient as temperatures measured at 24 h were similar in all groups of mice.

### 3.2 Induction of hypoglycemia by 145-2C11 mAb

DBA/2 mice injected with intact 145-2C11 mAb developed a marked hypoglycemia which persisted 24 h after the injection (Table 1). A decrease in blood glucose levels was also observed 6 h after the injection of $F(ab')_2$ fragments of 145-2C11 mAb. However, this hypoglycemia was significantly less intense than after administration of the intact

**Table 1.** Blood glucose levels after injection of 145-2C11 mAb

| Antibody preparation | *n* | Blood glucose levels (mg/100 ml)[a] 6 h | 24 h |
|---|---|---|---|
| Intact 145-2C11 | 11 | 76 ± 13[b] | 92 ± 22[b] |
| $F(ab')_2$ 145-2C11 | 5 | 109 ± 14[c] | 159 ± 17 |
| Control[c] | 13 | 140 ± 12 | 153 ± 21 |

a) Measured 6 h and 24 h after injection of 145-2C11 (mean ± SD); normal values in uninjected mice were 147 ± 24 mg/100 ml ($n = 56$).
b) $p < 0.001$ as compared with $F(ab')_2$ 145-2C11 and control hamster IgG.
c) IgG purified from normal hamster serum.
c) $p < 0.001$ as compared with control.

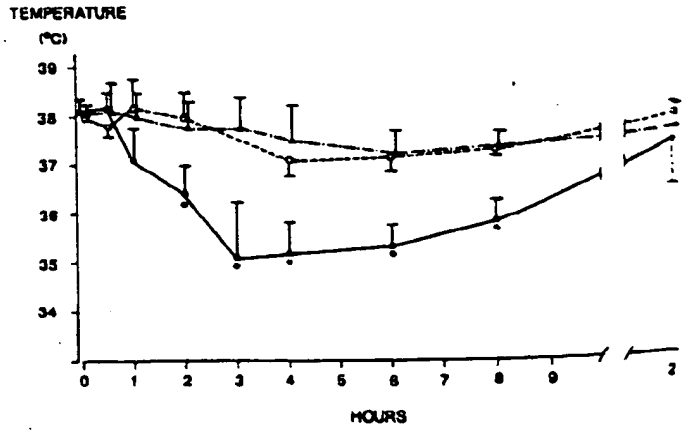


*Figure 1.* Rectal temperature in DBA/2 mice injected either with 50 µg of intact 145-2C11 (●—●), 50 µg of control hamster IgG (Δ—Δ) or 33 µg of $F(ab')_2$ fragments of 145-2C11 (○—○). Each point represents the mean (± SD) of 5 to 32 mice. * $p < 0.01$ as compared with mice injected with control hamster IgG or with $F(ab')_2$ 145-2C11.

mAb and was transient since blood glucose levels had returned to normal values at 24 h. On the other hand, blood glucose levels remained stable after injection of control hamster IgG and similar to those of uninj cted mice (147 ± 24 mg/100 ml).

### 3.3 TNF serum levels after injection f 145-2C11 mAb

As shown in Fig. 2, intact 145-2C11 results in an early and marked release of TNF in the circulation, maximal 2 h after the injection (peak TNF serum levels at 2 h: 50 ± 23 pg/ml). In contrast, the changes in serum TNF after injection of $F(ab')_2$ fragments of 145-2C11 were of low magnitude and similar to those detected after administration of control hamster IgG (Fig. 2). The activities detected in this assay could be completely neutralized by rabbit antibodies specific for murine TNF-α (data not shown).

In order to verify that the release of TNF induced by the 145-2C11 preparation depends on the action of the mAb on T cells, we compared the peak serum levels of TNF obtain d in *nu/nu* and in *nu/+* mice. No rise in serum TNF was observed in athymic *nu/nu* mice while a significant increase was detected in the *nu/+* counterparts (Table 2).

**Table 2.** Lack of TNF release after injection of intact 145-2C11 mAb in nude mice

| Mouse strain | *n* | Peak levels of TNF[a)] |
|---|---|---|
| *nu/nu* | 7 | 0.2 ± 0.3[b)] |
| *nu/+* | 7 | 14.6 ± 5.7 |

a) Measured 2 h after injection of the mAb and expressed as pg/ml (mean ± SD).
b) $p < 0.001$ as compared with *nu/+* mice.

As an additional control, the 145-2C11 mAb was injected in LPS resistant C3H/HeJ mice. The high TNF serum levels measured 2 h after the injection (59 ± 44 pg/ml) confirmed that the release of TNF induced by the 145-2C11 mAb did not depend on th presence of LPS in the antibody preparation.

### 3.4 Effects f anti-TNF antibodies n the metabolic changes induced by 145-2C11 mAb

The role of TNF in the metabolic changes induced by 145-2C11 was investigated by pretreating mice with rabbit anti-murine TNF antibodies before the injection of the mAb. As shown in Fig. 3, these mice were protected against the hypothermia induced by 145-2C11 mAb while animals pretreated with control rabbit IgG were not. Neutralization of TNF appeared somewhat less efficient to prevent the hypoglycemia since blood glucose levels in mice pretreated with anti-TNF antibodies remained significantly below the normal values (147 ± 24 mg/100 ml) both 6 h (88 ± 10 mg/100 ml, $p < 0.001$) and 24 h (123 ± 13 mg/100 ml, $p < 0.001$) after injection of 145-2C11. However, the

**Table 3.** Effect of TNF neutralization on the hypoglycemia induced by 145-2C11 mAb

| Pretreatment[a)] | *n* | Blood glucose levels (mg/dl)[a)] 6 h | 24 h |
|---|---|---|---|
| Anti-TNF antibodies | 7 | 88 ± 10[b)] | 123 ± 13[b)] |
| Control rabbit IgG | 5 | 59 ± 8 | 90 ± 14 |

a) Rabbit anti-TNF antibodies or control rabbit antibodies (12 mg/mouse) were injected i.v. 90 min prior to the administration of 50 µg 145-2C11 mAb.
b) $p < 0.01$ as compared with mice pretreated with control rabbit IgG.

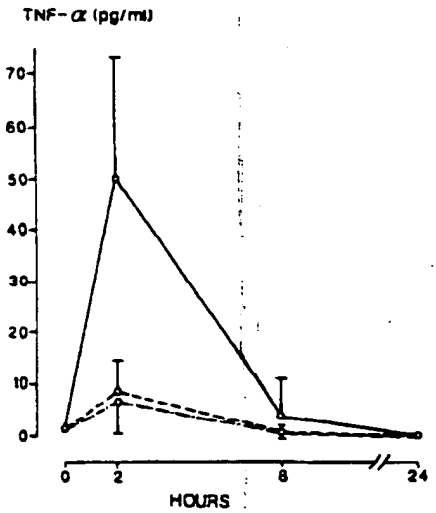


*Figure 2.* Serum TNF levels in the h urs following the injection of either intact 145-2C11 (—; 50 µg), control hamster IgG (– – –; 50 µg) r $F(ab')_2$ fragments of 145-2C11 (– · – · ; 33 µg) into DBA/2 mice. Each point represents the mean (± SD) of five to eight mice. * $p < 0.01$ as compared with mice injected with control hamster IgG or with $F(ab')_2$ fragments of 145-2C11.

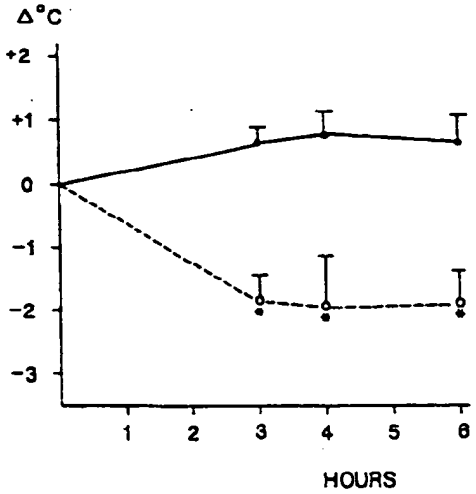


*Figure 3.* Prevention of the hypothermia induced by 145-2C11 mAb by rabbit anti-murine TNF mAb (●—●) and control rabbit IgG (O—O). (Of these antib dies 12 mg were administered i.v. 90 min before the injection of 145-2C11.) Each point represents the mean (± SD) of five to ten mice. * $p < 0.01$ as compared with mice pretreated with control rabbit Ig.

magnitude of hypoglycemia was significantly attenuated by the pretreatment with anti-TNF antibodies (Table 3).

## 4 Discussion

The administration of anti-CD3 mAb in mice results in a significant morbidity, as it does in man [2, 3]. Thus, Hirsch et al. reported the occurrence of anorexia, diarrhea, splenomegaly after a single injection of 145-2C11 as well as fatal anaphylactic reactions after multiple injections of the mAb [4]. In the present study, we observed acute hypothermia and hypoglycemia as additional side-effects of this mAb. Since similar changes have been detected after infusion of recombinant rTNF [13, 14], we performed serial determinations of serum TNF levels after injection of the mAb. These experiments showed that the 145-2C11 indeed induces the release of TNF into the circulation. This phenomenon does not depend on the presence of LPS in the preparation as it is observed in LPS-resistant C3H/HeJ mice but not in athymic nude mice which are LPS sensitive [15]. Both T cells and monocytes produce TNF after *in vitro* stimulation of human PBML with OKT3 [16] but the cellular source of the TNF released after *in vivo* administration of anti-CD3 mAb has not been determined so far. The absence of TNF release in nude mice indicates that 145-2C11 must interact with mature T cells to trigger the production of TNF. The fact that only low levels of serum TNF were observed after administration of $F(ab')_2$ fragments of 145-2C11 mAb suggests that binding of the Fc portion of the antibody on FcR-bearing cells is also required for the induction of TNF release. This is in keeping with the findings of Hirsch et al. who reported that only the intact form of 145-2C11 induces the secretion of CSF as a consequence of T cell activation *in vivo* [9].

The hypothermia induced by 145-2C11 appears to be mediated by TNF since it can be completely prevented by passive immunization with anti-TNF antibodies. The diarrhea is another side-effect of the mAb which is abolished by TNF neutralization (data not shown). The effects of anti-TNF antibodies on blood glucose levels indicate that TNF plays also a role in the hypoglycemia induced by 145-2C11. However, the mechanisms of the hypoglycemic effect are probably more complex than those of hypothermia. First, a transient hypoglycemia is also observed after injection of $F(ab')_2$ fragments of 145-2C11 whereas this preparation does not induce neither hypothermia nor significant TNF release. Second, neutralization of TNF has only a partial effect on the changes in blood glucose levels. Taken together, these data suggest that TNF is not the only mediator of the hypoglycemia induced by 145-2C11.

The OKT3 mAb as well as polyclonal anti-thymocyte globulin have been found to induce the release of high amounts of TNF into the circulation of kidney transplant recipients, with kinetics very similar to what we observed after injection of 145-2C11 in mice [6, 7, 17]. Our findings may thus be relevant for the understanding and the prevention of the side-effects of anti-lymphocyte antibodies in man. Indeed, several f th sympt ms observed aft r the first injection f OKT3 in recipients of organ allografts could b m diated by TNF, such as chills, fever, headache, myalgias and nausea [18]. It is, therefore, possible that inhibition of TNF release or n utralization of TNF by appropriate mAb would improve the clinical tolerance of OKT3. However, one should keep in mind that other cytokines such as IFN-γ, IL 2, CSF and IL 6 are also released after injection of anti-CD3 mAb [9, 19] and that some of these mediators may by themselves induce systemic symptoms [20] and xert detrimental effects on the transplanted organ [21]. In this respect, the use of $F(ab')_2$ fragments of anti-CD3 mAb may be of interest as they could allow to obtain immunosuppression without inducing the full-blown T cell activation leading to the massive release of cytokines.

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*Brief Definitive Report*

# In Vivo Induction of Interleukin 10 by Anti-CD3 Monoclonal Antibody or Bacterial Lipopolysaccharide: Differential Modulation by Cyclosporin A

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## Summary

We investigated the in vivo effects of cyclosporin A (CsA) on the production of interleukin (IL) 10, a cytokine with major immunosuppressive properties. To elicit IL-10 production in vivo, BALB/c mice were injected either with the anti-mouse CD3 145-2C11 monoclonal antibody (mAb) (25 μg) or with bacterial lipopolysaccharide (LPS) (20 μg). A systemic release of IL-10 was observed in both models, IL-10 serum levels reaching 1.60 ± 0.32 U/ml (mean ± SEM) and 0.67 ± 0.09 U/ml 6 h after injection of 145-2C11 mAb and LPS, respectively. Experiments in nude mice indicated that T cells are involved in the induction of IL-10 by anti-CD3 mAb, but not by LPS. Pretreatment with CsA (total dose: 50 mg/kg) before injection of 145-2C11 mAb completely prevented the release of IL-10 in serum as well as IL-10 mRNA accumulation in spleen cells. In contrast, CsA markedly enhanced LPS-induced IL-10 release (IL-10 serum levels at 6 h: 8.31 ± 0.43 vs. 0.71 ± 0.15 U/ml in mice pretreated with CsA vehicle-control, $p < 0.001$), as well as IL-10 mRNA accumulation in spleen. We conclude that CsA differentially affects IL-10 production in vivo depending on the nature of the eliciting agent. This observation might be relevant to clinical settings, especially in organ transplantation.

A major property of IL-10 is to inhibit cell-mediated immunity by blocking several functions of APCs, including the delivery of accessory signals to $CD4^+$ Th cells of the TH0 or TH1 type (1–3). It is therefore anticipated that IL-10 might play an important regulatory role in the process of allograft rejection. As cyclosporin A (CsA) is currently used in most immunosuppressive protocols in organ transplantation, we were interested in determining the effects of this drug on IL-10 production in vivo.

Since in vitro studies established that in addition to $CD4^+$ cells, macrophages and B cells also represent potential sources of IL-10 (4–6), two different stimuli were used to induce IL-10 production in mice: the 145-2C11 anti-mouse CD3 mAb as a polyclonal T cell activator, and bacterial LPS as an activating agent for B cells and macrophages. In both settings, increased serum levels of IL-10, as well as IL-10 mRNA accumulation in spleen were observed. These two parameters were therefore used to study the in vivo modulation of IL-10 production by CsA.

## Materials and Methods

*Mice.* 6–8-wk-old BALB/c mice and nude mice were obtained from the Katholieke Universiteit of Leuven (Leuven, Belgium) and from Olac (Bicester, England), respectively.

*Agents Injected In Vivo.* The hamster mAb 145-2C11 directed against the mouse CD3 complex (7) was prepared from culture supernatants of 145-2C11 hybridoma cells by affinity chromatography over a protein A-Sepharose column (Pharmacia, Uppsala, Sweden), as previously described (8). Control hamster Igs were purified from normal hamster serum by the same procedure. The endotoxin levels of these preparations were <15 pg/ml. CsA and its vehicle for parenteral administration were a kind gift of Sandoz Ltd. (Basel, Switzerland). LPS from *Escherichia coli* was obtained from Sigma Chemical Co. (St. Louis, MO).

*Experimental Protocols.* Mice received a single intraperitoneal injection of either 145-2C11 mAb (25 μg) or control hamster Igs (25 μg), or LPS (20 μg). Blood samples were obtained by retro-orbital puncture at 1.5, 6, 12, and 24 h after injection for measurement of serum IL-10 levels. In separate experiments, mice were killed

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2 h after injection for IL-10 mRNA determination in spleen cells. The effects of CsA on the induction of IL-10 by anti-CD3 mAb or LPS were evaluated by pretreating mice with two 25-mg/kg i.p. injections of CsA (or its vehicle alone as control) given 18 and 3 h before anti-CD3 mAb or LPS challenge. This protocol was found previously to prevent the release of IL-4 induced by the 145-2C11 mAb (9).

*Determination of IL-10 Serum Levels by ELISA.* Serum samples were assayed for IL-10 by ELISA (10) using the following anti-mouse IL-10 mAbs: SXC1, a rat IgM kindly provided by Dr. T. Mosmann (University of Alberta, Edmonton, Canada), and JES5-2A5, a rat IgG1 obtained from Pharmingen (San Diego, CA). Briefly, 96-well trays were coated with SXC1 mAb (5 μg/ml in PBS) during a 3-h incubation at 37°C. Excess protein binding sites were blocked by a further incubation with 2% BSA in PBS. Serum samples diluted in PBS containing 0.5% BSA were then added and incubated overnight at 4°C. After washing, the JES5-2A5 mAb (50 ng/well) was incubated for 2 h at 37°C. Bound JES5-2A5 mAb was revealed by a biotinylated mouse anti-rat IgG1 mAb (Experimental Immunology Unit, Université Catholique de Louvain) followed by streptavidin-horseradish peroxidase conjugate. Results were expressed in IL-10 U by reference with a standard curve obtained with a preparation of recombinant mouse IL-10 (Pharmingen). The lower limit of detection of IL-10 in this assay was 0.5 U/ml. For calculation of mean ± SEM values, samples below that threshold were arbitrarily assigned a value of 0.4 U/ml.

*Reverse PCR for IL-10 mRNA.* RNA extraction from mouse spleen cells using the guanidinium thiocyanate method, preparation of cDNA and PCR for IL-10 and hypoxanthine phosphoribosyl transferase (HPRT) were performed by standard procedures (11). Briefly, 1 μg of total RNA was incubated 10 min at 65°C with 1 μg oligo(dT)15 and was further incubated for 60 min at 37°C with 120 U RNasin (Promega Corp., Madison, WI), 1 mM dNTPs, 200 U Moloney murine leukemia virus (Mo-MuLV) reverse transcriptase, 0.01 mg/ml acetylated BSA, and RT buffer (75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 50 mM Tris HCl, pH 8.3) in a final volume of 20 μl. PCR was performed using aliquots of the resulting cDNA (equivalent to 50 and 500 ng of total RNA for HPRT and IL-10 assays, respectively). To this was added 0.1 mM dNTPs, 2.5 U Taq DNA polymerase, 1 μg of each sense/antisense primer, and PCR buffer (1.2 mM $MgCl_2$, 50 mM KCl, 0.001% gelatine, 10 mM Tris HCl, pH 8.3) in a total volume of 25 μl. Primers used have been published (2, 12). Reactions were incubated in a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) for 28 cycles (denaturation: 1 min, 93°C; annealing: 2 min, 55°C; extension: 3 min, 72°C). PCR products were run on a 3% agarose gel and stained with ethidium bromide.

*Statistical Analysis.* Statistical comparisons were made using the unpaired Student's *t* test. In each group, values from all mice were included.

## Results

*Release of IL-10 in Serum after Injection of Anti-CD3 mAb or LPS into BALB/c Mice.* Whereas serum from normal BALB/c mice did not contain detectable IL-10 as assayed by ELISA (lower limit of detection: 0.5 U/ml), the injection of 25 μg of the anti-CD3 mAb 145-2C11 was followed by the appearance of circulating IL-10 (Fig. 1, *top*). Serum IL-10 was already detected at 1.5 h in three out of eight mice (mean ± SEM: 0.70 ± 0.19 U/ml), peaked at 6 h (mean ± SEM: 1.60 ± 0.32 U/ml), and returned to near background values at 24 h. This release of IL-10 was directly related to the antibody specificity of the 145-2C11 mAb as it was not observed in mice injected with control hamster Igs (Fig. 1, *top*).

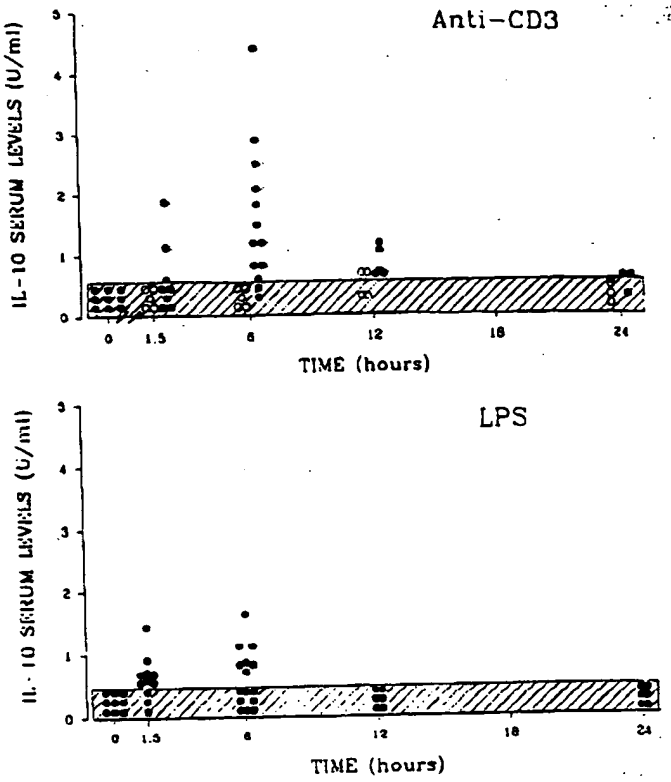


**Figure 1.** Release of IL-10 in serum after a single injection of 145-2C11 anti-mouse CD3 mAb or bacterial LPS in BALB/c mice. (*Top*): (●) mice injected with 25 μg 145-2C11 mAb; (○) controls injected with 25 μg of control hamster Igs. (*Bottom*) Mice injected with 25 μg LPS.

The injection of LPS (25 μg) into BALB/c animals was also followed by the systemic release of IL-10 (Fig. 1, *bottom*), although IL-10 serum levels were lower than after injection of the 145-2C11 mAb. IL-10 was detectable in serum 1.5 h after LPS injection in 8 out of 11 mice (mean ± SEM: 0.68 ± 0.09 U/ml), and at 6 h in 7 out of 15 mice (mean ± SEM: 0.67 ± 0.9 U/ml). No serum IL-10 was found at later time points.

*CsA Prevents Anti-CD3 mAb-induced Production but Enhances LPS-induced IL-10 Production.* The in vivo effects of CsA on the systemic release of IL-10 were investigated in animals pretreated with two 25-mg/kg i.p. injections of CsA given 18 and 3 h before challenge with anti-CD3 mAb or LPS (Fig. 2). CsA completely prevented the systemic release of IL-10 induced by anti-CD3 mAb as indicated by the lack of detectable serum IL-10 at all time points studied. As control, mice were pretreated with CsA vehicle alone before anti-CD3 mAb injection. The IL-10 serum levels in these animals were similar to those measured in the absence of pretreatment (mean ± SEM at 6 h: 1.69 ± 0.46 U/ml). In contrast with its inhibitory effect on anti-CD3 mAb-induced IL-10 production, CsA pretreatment led to a major increase in the IL-10 serum levels measured 6 h after LPS challenge (mean ± SEM: 8.31 ±

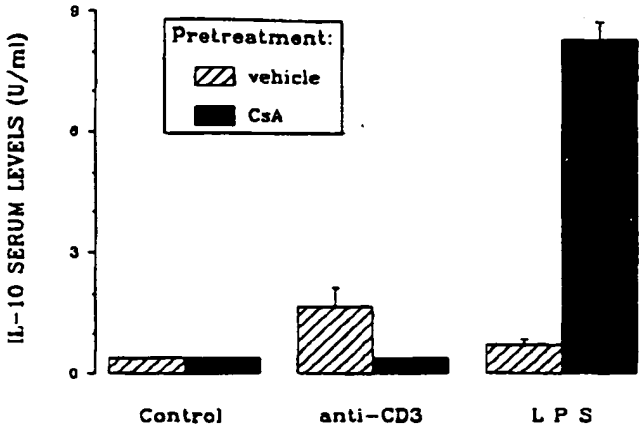


**Figure 2.** Effects of CsA pretreatment on the systemic release of IL-10 induced by anti-CD3 mAb or LPS in BALB/c mice. Mice ($n$ = 5 in each group) were pretreated with two 25 mg/kg-i.p. injections of CsA or CsA vehicle before injection of 25 μg 145-2C11 mAb or 20 μg LPS. IL-10 serum levels were measured 6 h after challenge, and data of all mice are presented as mean ± SEM.

0.43 U/ml vs. 0.71 ± 0.15 in mice pretreated with CsA vehicle alone, $p < 0.001$) (Fig. 2).

We analyzed whether the effects of CsA on IL-10 release were related to modulation of IL-10 gene expression. No or minimal expression of IL-10 mRNA was found in spleens of untreated mice or of mice injected with CsA alone (Fig. 3). Anti-CD3 mAb injection led to a marked accumulation f IL-10 mRNA which was almost completely prevented by CsA pretreatment (Fig. 3). LPS also led to IL-10 gene induction and in this setting CsA pretreatment resulted in a clear increase in IL-10 mRNA accumulation (Fig. 3).

*LPS but Not Anti-CD3 mAb Induces IL-10 Production in Nude Mice.* The role of T cells in IL-10 production induced by anti-CD3 mAb or LPS was investigated by challenging nude mice with these stimuli. Neither unmanipulated nude mice

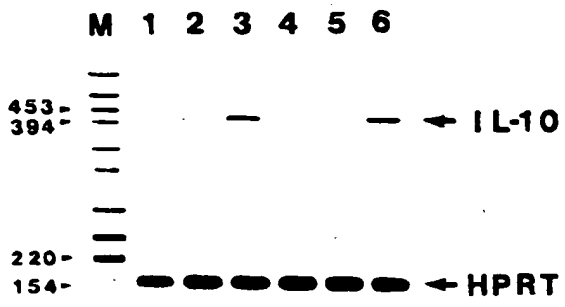


**Figure 3.** Effects of CsA pretreatment on IL-10 mRNA expression in spleen after injection of 145-2C11 anti-CD3 mAb or LPS in BALB/c mice (same protocol as in Fig. 2). Spleens were removed 2 h after anti-CD3 mAb or LPS challenge and analyzed by reverse PCR for IL-10 mRNA expression. Amplification of the housekeeping gene HPRT was used as control. (Lane *1*) Control mice injected with saline; (lane *2*) CsA alone; (lane *3*) anti-CD3 mAb; (lane *4*) anti-CD3 mAb after CsA pretreatment; (lane *5*) LPS; lane *6*: LPS after CsA pretreatment.

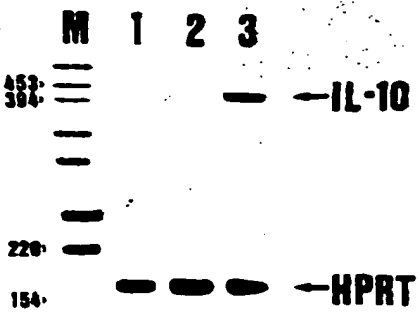


**Figure 4.** IL-10 mRNA expression in spleens of nude mice after injection of 145-2C11 anti-CD3 mAb or LPS. Spleens were removed 2 h after anti-CD3 mAb or LPS challenge and analyzed by reverse PCR for IL-10 and HPRT mRNA expression. (Lane *1*) Control mice injected with saline; (lane *2*) anti-CD3 mAb; (lane *3*) LPS.

nor nude mice injected with anti-CD3 mAb displayed significant IL-10 gene transcription (Fig. 4). IL-10 also remained undetectable in their serum at all time points. On the other hand, as in BALB/c mice, LPS injection led to IL-10 mRNA accumulation (Fig. 4) and induced a systemic release of IL-10 (mean ± SEM at 6 h: 0.63 ± 0.10 U/ml, $n$ = 5), which was further increased by CsA pretreatment (mean ± SEM at 6 h: 4.82 ± 1.35 U/ml, $n$ = 5, $p < 0.05$ as compared with LPS alone).

## Discussion

The first observation of this study is that both anti-CD3 mAb and LPS induce the release of immunoreactive IL-10 in the bloodstream of normal mice. The range of IL-10 serum levels in both settings was quite large, with some samples remaining below the detection limit. Such wide dispersion of cytokine serum levels has been previously observed in similar models (13, 14). It is likely that the cell types involved in IL-10 production after injection of anti-CD3 mAb or LPS are different. Indeed, experiments in nude mice established that T cells are required for anti-CD3 mAb–induced but not for LPS-induced IL-10 production. As previously demonstrated for IL-2, IFN-γ, and IL-4 mRNAs (15), we might thus assume that T cells are the major source of IL-10 after injection of anti-CD3 mAb. Along this line, it was recently shown that the $CD4^+$ cell population was the cell type in which IL-10 mRNA accumulates after injection of anti-mouse IgD mAb (16). As far as LPS-induced IL-10 production is concerned, both macrophages and B cells, especially $Ly\text{-}1^+$ B cells, might be involved (5, 6). Whatever its precise cell source, IL-10 might be responsible for some of the immune disturbances observed after injection of anti-CD3 mAb or LPS. First, the long-lasting immunosuppressive effects of anti-CD3 mAbs which have been observed both in experimental and clinical transplantation (17, 18) could be related, at least in part, to the production of IL-10. The immunosuppression observed after LPS injection (19, 20) could also represent a consequence of IL-10 production. Experiments using neutralizing anti–IL-10 mAb r mice made IL-10–deficient by gene

targeting should help to define the exact role of IL-10 in the immunosuppression induced by anti-CD3 mAb and LPS.

The main findings reported in this paper concern the differential effects of CsA on the production of IL-10 induced by anti-CD3 mAb or LPS. Although it has been well established that CsA inhibits in vitro and in vivo the transcription of several cytokine genes in mouse T cells including IL-2, IFN-γ, and IL-4 genes (9, 21, 22), it was recently reported that the drug does not affect the production of IL-10 by a TH2 clone (23). The experiments described herein clearly establish that CsA blocks the systemic release of IL-10, as well as IL-10 gene expression induced by anti-CD3 mAb in vivo. In contrast, CsA pretreatment in the LPS model resulted in the superinduction of IL-10 gene expression and in a dramatic enhancement in the systemic release of IL-10. Similar gene superinduction by CsA has been previously reported for TGF-β in human T cells (24), for IL-6 in human PBMCs (25), and for the Ly-6E surface antigen in the T cell lymphoma–derived YAC-1 cell line (26). Mechanisms proposed to explain CsA-mediated cytokine gene superinduction include the inhibition of the production of nuclear factors binding to negative regulatory sequences of corresponding gene or the lack of inhibition of regulatory proteins that promote cytokine gene expression (24, 26). Our experiments in nude mice indicate that the enhancement by CsA of LPS-induced IL-10 gene expression and IL-10 production does not depend on the action of the drug on T cells and could therefore be related to a direct effect on IL-10–producing cells.

The in vivo effects of CsA on IL-10 production might be relevant to clinical settings where CsA is used to induce immunosuppression, such as in organ transplantation or in autoimmune diseases. Administration of CsA in transplant recipients receiving the OKT3 mAb has been recommended to reduce the antibody response to the mAb. However, by blocking OKT3-induced IL-10 production, CsA might also inhibit an important pathway of immunosuppression. In patients developing endotoxemia under CsA therapy, the enhanced IL-10 production might be beneficial by reducing the release of TNF-α and IL-1 (27), but detrimental by accentuating the Th cell defects induced by LPS.

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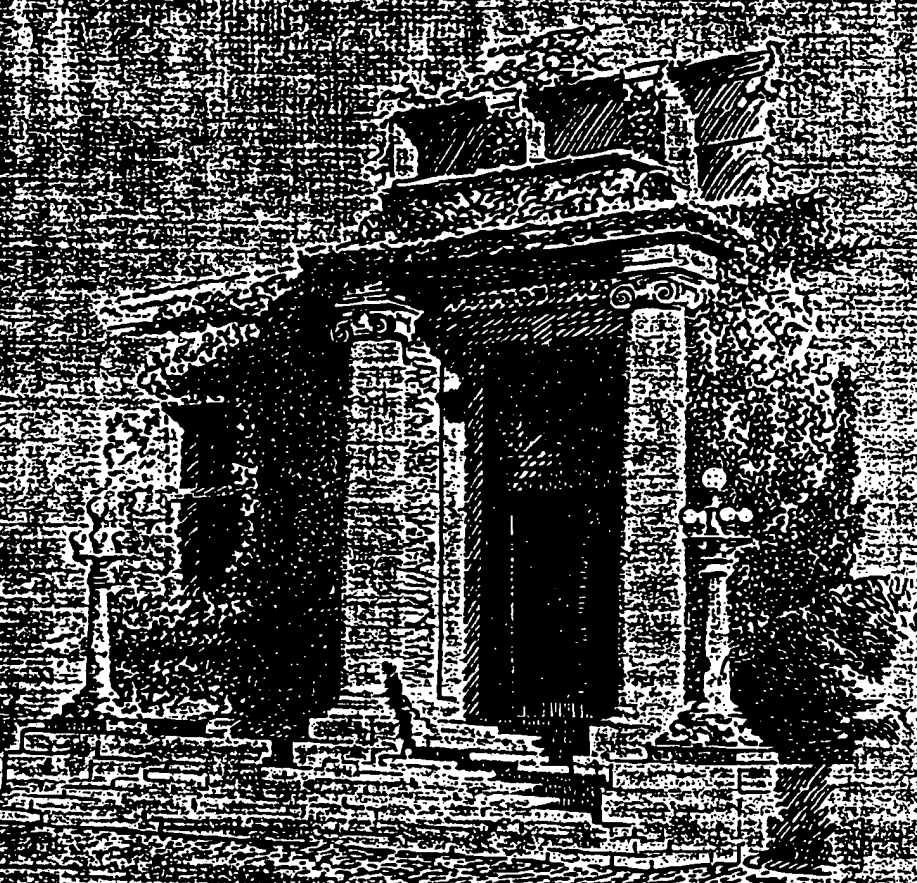

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# CD4$^{pos}$, NK1.1$^{pos}$ T C lls Promptly Produce Interleukin 4 in Response to In Vivo Challenge with Anti-CD3

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**Summary**

Injection of anti-CD3 antibodies causes prompt expression of interleukin (IL)-4, IL-2, and interferon $\gamma$ (IFN-$\gamma$) mRNA among spleen cells. The optimal dose of anti-CD3 for such induction was 1.33 $\mu$g/animal; lymphokine mRNA was first observed at 30 min, peaked at 90 min, and was undetectable (for IL-4) or had declined markedly by 4 h. Cells harvested from spleens of mice injected with anti-CD3 90 min earlier secreted IL-4, IL-2, and IFN-$\gamma$ without further stimulation. By contrast, in vitro stimulation with anti-CD3 of spleen cell suspensions or splenic fragments from noninjected donors failed to cause prompt production of IL-4 and, even after 24 h of stimulation, the amount of IL-4 produced in such cells was substantially less than that secreted within 1 h by spleen cell suspensions or splenic fragments from mice injected with anti-CD3 90 min earlier. Production of IL-4 by spleen cells from anti-CD3–injected mice was not inhibited by pretreatment with anti-IL-4 antibody or with IFN-$\gamma$ or tumor growth factor $\beta$ nor enhanced by treatment with IL-4. By contrast, CTLA-4 immunoglobulin (Ig) treatment clearly diminished IL-4 production in response to in vivo anti-CD3, indicating that cellular interactions involving CD28 (or related molecules) were important in stimulation. Cell sorting analysis indicated that the cells that produced IL-4 in response to in vivo injection of anti-CD3 were highly enriched in CD4$^{pos}$ cells with the phenotype leukocyte cell adhesion molecule-1 (LECAM-1)$^{dull}$, CD44$^{bright}$, CD45RB$^{dull}$, NK1.1$^{pos}$. Indeed, the small population of CD4$^{pos}$, NK1.1$^{pos}$ cells had the great majority of the IL-4–producing activity of this population. Injection with *Staphylococcal* enterotoxin B also caused prompt induction of IL-4 mRNA; the cells that were principally responsible for production also had the phenotype of CD4$^{pos}$, NK1.1$^{pos}$. These results suggest that possibility that this rare population of T cells may be capable of secreting IL-4 at the outset of immune responses and thus may act to regulate the pattern of priming of naive T cells, by providing a source of IL-4 to favor the development of T cell helper 2–like IL-4–producing cells.

Immune responses of CD4$^{pos}$ T cells are often dominated by the production of IFN-$\gamma$ or of IL-4. Indeed, the choice of lymphokine produced by CD4$^{pos}$ T cells may have a profound effect on the protective value of that response (1–3). Recent in vitro studies have analyzed the factors that determine whether naive CD4$^{pos}$ T cells develop into IFN-$\gamma$ or IL-4 producers (4–8). These studies have revealed a dominant role for IL-4 itself. Thus, priming T cells in the presence of IL-4 leads to the appearance of cells that produce large amounts of IL-4 upon rechallenge but little or no IFN-$\gamma$ (6, 7). By contrast, priming cells in the presence of anti-IL-4 antibody results in primed cell populations that produce little or no IL-4 but are good IFN-$\gamma$ producers. For IL-4 to have a major effect on priming for IL-4 production, it must be present early in the culture.

IL-4 also has a major effect on in vivo priming of CD4$^{pos}$ T cells to become IL-4–producing cells (9–12). Treatment of mice with anti-IL-4 at the time of priming with hemocyanin diminishes the frequency of T cells that produce IL-4 in response to in vitro challenge with hemocyanin (11). Similarly, treatment of BALB/c mice with anti-IL-4 at the time of infection with *Leishmania major* (9) or *Candida albicans* (10) enhances their production of IFN-$\gamma$ and suppresses priming for IL-4 production in response to specific challenge, but only if anti-IL-4 is injected very early in the course of the infection. Treating C57BL/6 mice with IL-4 at the time of infection with *L. major* increases the appearance of T cells that produce IL-4 upon subsequent in vitro challenge with leishmanial antigens (12). CD4$^{pos}$ T cells from mice in which the IL-4 gene has been disrupted by gene targeting produce substantially less IL-5 as a result of infection with *Nippostrongylus brasiliensis* (13). Since IL-5 and IL-4 are both products of Th2-type T cell clones (14) and their pr duction by normal T cells is often coregulated (5), these results further support

the concept that IL-4 plays a critical role in the in vivo acquisition of the lymphokine-producing phenotype. Taken together, these studies indicate that IL-4 produced early in an immune response is important in determining th pathway of differentiation of naive $CD4^{pos}$ T cells.

The central role played by IL-4 in in vivo priming leads us to ask what the source of IL-4 might be for determining that naive $CD4^{pos}$ T cells will develop into IL-4 producers. Although T cells themselves are an obvious possibility, the very modest production of IL-4 by T cell populations from naive donors when stimulated in vitro has cast some doubt on their potential importance and raised the possibility that other cell types, such as basophils and/or mast cells, might be the source of such IL-4 (15–18). Nonetheless, both Scott et al. (19) and Flamand et al. (20) have observed that intravenous injection of soluble anti-CD3 antibody results in rapid but transient expression of IL-4 mRNA and protein in the spleen. In this report, we confirm this observation and show that IL-4 protein is induced as early as 30 min after the injection of anti-CD3 antibody. It is interesting to note that in vitro treatment of spleen cells with anti-CD3 in either suspension or fragment cultured led to induction of modest amounts of IL-4 protein with much slower kinetics indicating that in vivo and in vitro regulation of IL-4 production may be quite different.

We show that the cells responsible for IL-4 production in response to intravenous anti-CD3 are largely $CD4^{pos}$ T cells. The $CD4^{pos}$ T cells that produce IL-4 in response to intravenous injection of anti-CD3 are leukocyte cell adhesion molecule (LECAM)[1]-$1^{dull}$,$CD44^{bright}$,$CD45RB^{dull}$. IL-4–producing cells are enriched among $CD4^{pos}$, $NK1.1^{pos}$ cells suggesting that the major producers are the small population of $CD4^{pos}$, $NK1.1^{pos}$ T cells that have been previously implicated as important lymphokine-producing cells in the thymus and bone marrow (21). The production of IL-4 by this cell population is independent of the need for IL-4 since anti-IL-4 treatment does not inhibit it. Such production of IL-4 is also mediated by intravenous injection of *Staphylococcal* enterotoxin B (SEB) suggesting an in vivo mechanism through which superantigens may elicit a source of IL-4 that could influence priming to associated conventional antigens.

## Materials and Methods

*Animals.* Virus-free C57BL/6 and BALB/c female mice, 8–12 wk of age, were obtained from the Division of Cancer Treatment, National Cancer Institute (Frederick, MD).

*Culture Medium.* RPMI 1640 (Biofluids, Inc., Rockville, MD) supplemented with 10% FCS (Inovar Biologicals, Inc., Gaithersburg, MD), 2-ME (50 μM), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and sodium pyruvate (1 mM) was used as culture medium.

*Recombinant Lymphokines.* Recombinant mouse IL-4 was obtained from a recombinant baculovirus (AcMNPV.IL-4) prepared by Cynthia Watson (Laboratory of Immunology, National Institute of Allergy and Infectious Diseases [NIAID], National Institutes of Health [NIH]). 1 U of IL-4 is equal to ~0.5 pg. Human recombinant IL-2 was a gift Cetus Corporation (Emeryville, CA). 1 U of IL-2, defined as a "Cetus unit", is equal to 6 IU and to ~0.3 ng. Purified mouse IFN-γ and TGF-β were purchased from Genzyme Corporation (Boston, MA).

*Reagents.* Anti-CD3 (2C11) (22) and control hamster monoclonal IgG antibody (HH16) (established by Carol Kinzer, Laboratory of Immunology, NIAID, NIH) were purified from tissue culture supernatants. Purified rat anti-mouse IL-4 (11B11) (23) was prepared by Verax Corporation (Lebanon, NH). Rat anti-mouse Fcγ receptor antibody (2.4G2) (24) was purified by Jane Hu-Li (Laboratory of Immunology, NIAID, NIH). Rat anti-mouse IFN-γ (XMG 1.2) (25), FITC-rat anti-mouse LECAM-1 (MEL-14) (26), FITC-rat anti-mouse CD44 (PgP-1) (27), FITC-rat anti-mouse CD45RB (16A) (28), FITC-rat anti-mouse B220 (RA3-6B2) (29), FITC-rat anti-mouse $I\text{-}A^d$ (AMS 32.1) (30), PE-rat anti-mouse CD4 (RM 4-5) (31), FITC-rat anti-mouse CD8 (53-6.7) (32), and biotinylated anti-mouse NK1.1 (PK 136) (33) were purchased from PharMingen (San Diego, CA). SEB was purchased from Sigma Chemical Co. (St. Louis, MO). A soluble IgCγ1 chimera of CTLA-4 (CTLA-4Ig) (34) and its control human–mouse chimeric mAb (Chi-L6) (35) were provided by Dr. Peter S. Linsley (Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA).

*In Vivo Treatment of Mice.* Mice were injected intravenously with a single dose of anti-CD3 (0.44–12 μg), or SEB (25–800 μg). Control mice were injected with same amount of hamster IgG or HBSS. Spleens were removed at the specified times after injection for RNA extraction and cell culture. For some experiments, mice were treated with lymphokines, anti-IL-4 or CTLA-4Ig before injecti n with anti-CD3. For the stimulation of popliteal lymph node cells, 10 μg of anti-CD3 in 50 μl of HBSS was injected into both hind foot pads of C57BL/6 mice. The draining popliteal lymph nodes were removed for RNA extraction at various times after injection.

*Cell Preparation.* Spleens were removed at various times after injection with anti-CD3 or SEB. Cell suspensions were washed twice with HBSS. Except where indicated, $5 \times 10^6$ spleen cells/well were cultured in 24-well plates with or without 3 μg/ml of anti-CD3 for 1–24 h and supernatants harvested to measure lymphokine content. In some experiments, spleens from mice injected with or without anti-CD3 were cut into cubes with a volume of ~1 $mm^3$. Individual cubes were placed in wells of 96-well plates and cultured with or without anti-CD3 for 1–24 h.

For the preparation of $CD4^{pos}$ splenic T cells, cells were suspended at a concentration of $2 \times 10^7$/ml in RPMI 1640 containing 5 mM EDTA (NIH Media Unit) and 5% fetal FCS. The cell suspension was incubated with 10 μg/ml each of FITC anti-B220; FITC anti-$I\text{-}A^d$, and FITC anti-CD8 antibodies for 30 min at 4°C on a turning wheel. The cells were then washed twice and resuspended with magnetic beads coated with sheep anti-FITC antibodies (Advanced Magnetics Inc., Cambridge, MA). Cells that had bound antibodies were depleted by two rounds of exposure to magnetic field. The residual cells were collected and washed twice.

*Fluorescence Analysis and Cell Sorting.* Fluorescence staining was performed at 4°C in 100 μl containing $10^6$ CD4-enriched spleen cells and PE anti-CD4 in combination with FITC anti-LECAM-1, FITC anti-CD44, or FITC anti-CD45RB in PBS containing 3% FCS and 0.5% $NaN_3$. For detection of $NK1.1^{pos}$ cells, biotinylated anti-NK1.1 and FITC-labeled avidin were used. Fluorescence analysis was carried out with a FACScan® Flow Cytometer (Becton Dickinson & Co., Mountain View, CA). For sorting experiments, a FACStar® Plus Flow Cytometer (Becton Dickinson & Co.) was used. Cells were maintained at 4°C during the sorting process.

*Lymphokine Assays.* CT.4S, an IL-4–dependent cell line, and

[1] *Abbreviations used in this paper:* LECAM, leukocyte cell adhesion molecule; RT, reverse transcriptase; SEB, *Staphylococcal* enterotoxin B.

CTLEV, an IL-2–dependent cell line (36), were used to measure IL-4 and IL-2 content, respectively, using serial dilutions of supernatants and comparing responses to those elicited by known amounts of murine IL-4 and human IL-2 as standards. IFN-$\gamma$ was assayed with a specific two-site ELISA (37, 38), with reference standard curves prepared using known amounts of rIFN-$\gamma$.

*Analysis of Expression of IL-4, IFN-$\gamma$, and IL-2 mRNA.* Total spleen RNA was prepared using the Guanidinium Method (39). In brief, after making spleen cell suspensions, cells were washed in ice-cold PBS and lysed in 4 ml lysis solution, containing 4 M guanidine-thiocyanate (Fluka Chemika-BioChemika, Ronkonkoma, NY), 25 mM sodium citrate (pH 7.0), 0.5% *N*-lauroyl sarcosine (Sigma Chemical Co.), and 100 mM 2-ME. Lysates were vortexed and stored at −70°C until further processing. After thawing, 400 $\mu$l of 2 M sodium acetate (pH 4.0), 4.4. ml of water-saturated phenol (AMRESCO, Solon, OH), and 800 $\mu$l of chloroform-iso-amyl alcohol (49:1) were added to the lysates with thorough vortexing after each addition. The mixture was then chilled on ice for 15 min and spun at 10,000 *g* for 15 min at 4°C. The aqueous phase was recovered and RNA was precipitated with an equal volume of 2-propanol at −20°C for at least 1 h. The precipitated RNA was resuspended with 600 $\mu$l of lysis solution and an equal volume of 2-propanol, and precipitated at −20°C for 1 h. Precipitates were pelleted at 4°C, washed twice in 70% ethanol, and repelleted at 4°C at 10,000 *g* for 20 min. Vacuum-dried pellets were resuspended in 50 $\mu$l of diethylpyrocarbonate-treated double-distilled water (DEPC-dd$H_2O$) and total RNA concentration was measured. As positive controls, mRNAs extracted from the IL-4–producing cell line, LT-1 (40) and from purified T cells stimulated with APC plus anti-CD3 and anti-IL-4 for 24 h (8) were used.

For RNA preparation from sorted cells, we used the modified method of Chomczynski and Sacchi (39) and resuspended pellets in 10–20 $\mu$l DEPC-dd$H_2O$.

For Northern hybridization, 10 $\mu$g of RNA from each sample were separated by electrophoresis in a 1% agarose/formaldehyde gel and blotted onto a nitrocellulose-Nytran membrane (Schleicher and Schuell, Inc., Keene, NH). cDNA probes specific for mouse IL-4, IFN-$\gamma$ and IL-2 (a 373 EcoRI/HindIII fragment for IL-4; a 643 bp Pst-1 fragment for IFN-$\gamma$; and a 600 bp Pst-1 fragment for IL-2) were $^{32}$P-labeled by the random primer method to a specific activity of $5 \times 10^8$–$2 \times 10^9$ cpm/$\mu$g. After baking, the filters were prehybridized at 42°C for 1 h and then hybridized with labeled probe for 18 h. The filters were washed twice with 2 × SSC and 0.1% SDS at room temperature and twice at 60°C with 0.1 × SSC and 0.1% SDS.

For analysis of expression of IL-4, IFN-$\gamma$, and IL-2 mRNA from sorted cells and in other selected experiments, mRNAs were amplified by a modified standard reverse transcription (RT)-PCR amplification procedure (41). Primers specific for murine IL-4, IL-2, IFN-$\gamma$, and $\beta$-actin were prepared by Cynthia Watson. Primer sequences were as follows: IL-4: 5′ primer, GAATGTACCAGCAGCCATATC, and 3′ primer, CTCAGTACTACGAGTAATCCA; IL-2: 5′ primer, ACTTCAAGCTCCACTTCAAGC, and 3′ primer, GCTTTGAGAAAGGGCTATCCA; IFN-$\gamma$: 5′ primer, AACGCTTACACACTGCATCTTGG, and 3′ primer, GACTTCAAAGAGTCTGAGG; $\beta$-actin: 5′ primer, GATGACGATATCGCTGCGCTG, and 3′ primer, GTACGACCAGAGGCATACAGG. Initially, 2 $\mu$l (1 $\mu$g) RNA were added to 18 $\mu$l of reverse transcription mixture containing 5 mM $MgCl_2$, 1× PCR buffer II, 1 mM of each dNTP (all from Perkin-Elmer Cetus, Norwalk, CT), 2,000 U/ml RNase inhibitor (Promega, Madison, WI), 2.5 U/ml M loney murine leukemia virus reverse transcriptase (GIBCO BRL, Gaithersburg, MD), and 0.75 $\mu$M specific 3′ oligo primer for each lymphokine. Tubes were then placed in a thermal cycler (GenAmp PCR System 9600; Perkin-Elmer Cetus) and incubated for 60 min at 37°C, followed by 5 min at 99°C, and then 5 min at 5°C.

After reverse transcription, 80 $\mu$l of the PCR mix were added t each tube to give a final concentration of 2.5 U Taq DNA polymerase (Perkin-Elmer Cetus), 0.15 $\mu$M 5′ primer, 0.15 $\mu$M 3′ primer, 2 mM $MgCl_2$, and 1× PCR buffer II/100 $\mu$l. cDNAs were amplified for 30 cycles, each composed of 94°C for 15 s, 55°C for 15 s, and 72°C for 1 min. At the end of 30 cycles, samples were stored at 4°C until analyzed. After amplification, PCR products were separated by electrophoresis in 8% acrylamide gels and visualized by UV light illumination after ethidium bromide staining. Some PCR products were analyzed by Southern blotting, using the probes described above.

## Results

*Expression of Lymphokine mRNA In Vivo.* C57BL/6 mice were injected intravenously with purified anti-CD3 (2C11) or control hamster antibody (HH16). Spleens were removed at various times after injection for RNA extraction. mRNAs for IL-4, IFN-$\gamma$, and IL-2 were measured by Northern analysis. After injection with 4 $\mu$g of anti-CD3, IL-4 mRNA was first detectable at 30 min, reached a higher level at 1 h, and was markedly diminished at 4 h. (Fig. 1 *A*). To determine the optimal dose of anti-CD3 for this induction of IL-4 mRNA, Northern analysis was carried out 1 h after injection with varying amounts of anti-CD3; 1.33 $\mu$g of anti-CD3 per mouse was found to maximally induce IL-4 mRNA.

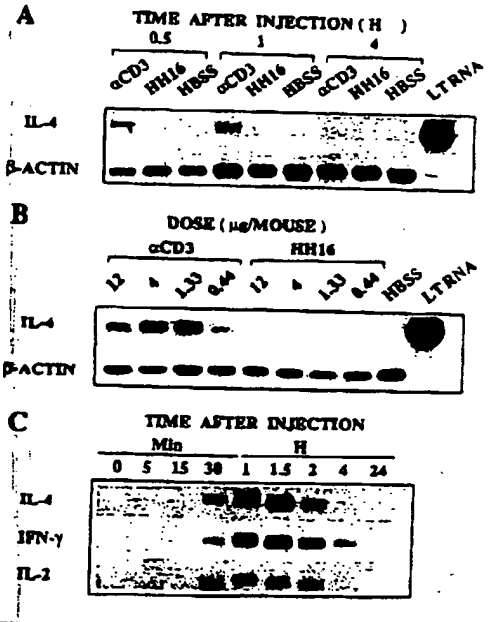


**Figure 1.** Anti-CD3 induces the expression of lymphokine mRNAs in vivo. C57BL/6 mice were injected with (*A*) 4 $\mu$g; (*B*) 0.44–12 $\mu$g; and (*C*) 1.33 $\mu$g of purified anti-CD3 (2C11), control hamster Ig (HH16), or HBSS, and spleens were harvested at the indicated time for RNA extraction. RNA from IL-4–secreting LT-1 cells (*LTRNA*) is included as a positive control for IL-4 mRNA.

expression (Fig. 1 *B*). In these experiments, th control hamster mAb did not induce detectable IL-4 mRNA.

To further examine the critical time for maximal induction of IL-4 mRNA, spleens were removed as early as 5 min after injection of 1.33 μg of anti-CD3. By Northern analysis, IL-4 mRNA was first detected at 30 min, reached its highest level at 1.5 h, and declined rapidly thereafter (Fig. 1 *C*). IFN-γ and IL-2 mRNAs were also initially expressed at 30 min and, although detectable at 4 h, had diminished considerably from their peak at 1.5 h. Based on these experiments, we used 1.33 μg of anti-CD3 per mouse and removed the spleen at 1.5 h after injection in the following experiments.

*Production of Lymphokines by Spleen Cells from Mice Treated with Anti-CD3.* To determine whether the expression of lymphokine mRNAs led to production of lymphokine, 5 × $10^6$ spleen cells from anti-CD3-injected mice were cultured for 1 h without additional stimulus. As shown in Fig. 2, a substantial amount of lymphokine was present in these supernatants; the time after in vivo injection to gain and then to lose the capacity to produce each of the lymphokines in short-t rm culture was the same as that for expression of mRNA at the time of harvest from the donor. Spleen cells removed 1.5 h after in vivo treatment produced maximal amounts of IL-4, IFN-γ, and IL-2. We measured lymphokine secretory activity as well as lymphokine mRNA in most of the following experiments.

*Organ Distribution of IL-4 mRNA Expression from Mice Injected with Anti-CD3.* In contrast to the prompt expression of IL-4 mRNA in spleen cells from mice injected intravenously with anti-CD3, little or no IL-4 mRNA was detected in thymocytes or peripheral blood cells of these mice (Fig. 3). Bone marrow cells and mesenteric lymph node cells showed modest amounts of IL-4 mRNA in response to injection with anti-CD3, but considerably less than was found in spleen cells.

Analysis of penetration of anti-CD3 into spleen, lymph nodes, and thymus was done by staining cells with FITC anti-hamster IgG and comparing the frequency of positive cells with that obtained by staining cells with anti-CD3 plus FITC anti-hamster IgG. Half of splenic T cells were stained within 15 min of injection of anti-CD3; neither the fraction nor the degree of staining increased for up to 90 min after injection (data not shown). By contrast, <10% of lymph node

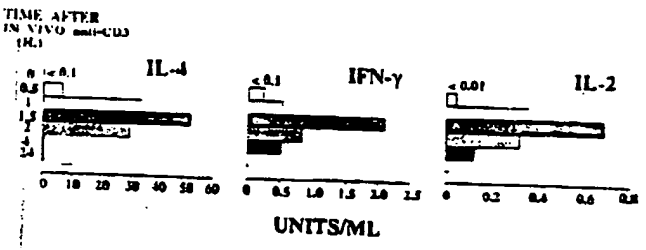


Figure 2. Production of lymphokines by spleen cells from mice treated with anti-CD3. Spleen cells from mice injected previously (as indicated) with 1.33 μg of anti-CD3 were cultured in 24-well plates at 5 × $10^6$/ml for 1 h without additional stimulation. Culture supernatants were harvested and tested for production of IL-4, IFN-γ, and IL-2.

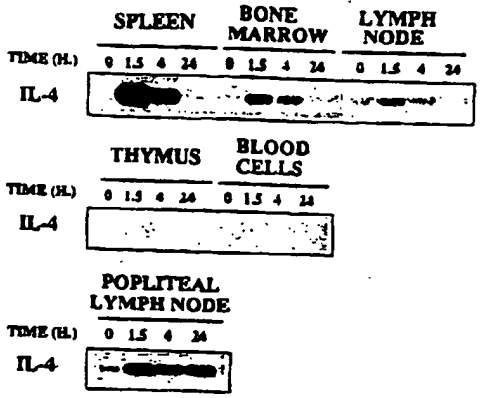


Figure 3. Organ distribution of IL-4 mRNA expression from mice injected with anti-CD3. RNA was extracted from spleen cells, bone marrow cells, mesenteric lymph node cells, thymocytes, and peripheral blood cells at various times after injection of 1.33 μg of anti-CD3. Popliteal lymph node cells were removed after injection of 10 μg of anti-CD3 in 50 μl of HBSS into hind foot pads. IL-4 mRNA levels were determined by RT-PCR with Southern blot analysis.

T cells and virtually none of the $CD3^{low}$ thymocytes had bound anti-CD3 at 90 min after injection. This suggests that limited (or absent) IL-4 production in these organs is due to poor penetration of injected anti-CD3.

Interestingly, popliteal lymph nodes from mice injected with anti-CD3 in the footpads displayed considerable IL-4 mRNA; peak levels were reached within 1.5 h but, in contrast to the spleen cells after intravenous treatment with anti-CD3, IL-4 mRNA among popliteal lymph nodes cells remained elevated for 24 h after footpad injection of anti-CD3.

*Comparison of IL-4 Production by Spleen Cells in Response to In Vivo and In Vitro Stimulation with Anti-CD3.* Spleen cells from mice treated with anti-CD3 for 1.5 h produce large amounts of IL-4 without further in vitro stimulation. By contrast, spleen cells from uninjected mice produced little or no IL-4 without in vitro stimulation and failed to produce substantial amounts of IL-4 in response to in vitro exposure to anti-CD3 until 24 h of culture (Fig. 4 *A*). Even at 24 h, the amount of IL-4 in culture supernatants of cells from uninjected donors cultured in vitro with anti-CD3 was less than the amounts of IL-4 in 1-h culture supernatants of spleen cells from mice injected with anti-CD3 1.5 h earlier and cultured without any further stimulant.

We also cultured splenic fragments from mice injected with anti-CD3 1.5 h earlier and from uninjected mice with or without additional anti-CD3 in vitro (Fig. 4 *B*). Fragments from the "1.5-h injected" mice produced substantial amounts of IL-4 within 1 h without the need for the addition of anti-CD3. Although splenic fragments from uninjected mice produced detectable amounts of IL-4 in response to culture with anti-CD3 somewhat earlier than was observed in spleen cell suspensions from these mice, this production was less in magnitude and still much delayed in comparison to the IL-4 production by splenic fragments from mice injected with anti-CD3 but not further cultured with anti-CD3. These results

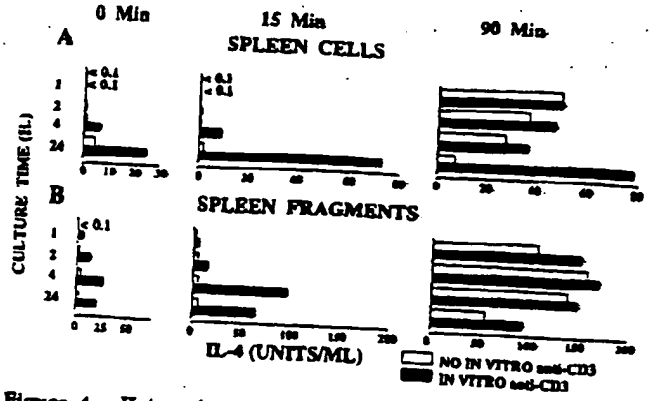


**Figure 4.** IL-4 production by spleen cells or splenic fragments in response to in vivo and in vitro stimulation with anti-CD3. Spleen cells (*A*) or spleen fragments (*B*) from uninjected mice (*left*) or from mice injected with anti-CD3 15 min (*middle*) or 90 min (*right*) earlier were cultured with (■) or without (□) 3 μg/ml of anti-CD3 for 1–24 h. Spleen cells suspensions were cultured at 2 × $10^5$ cells in 0.2 ml of culture medium in 96-well plates; single spleen fragments with a volume of ~1 $mm^3$ were cultured in 0.2 ml in individual wells of 96-well plates. Supernatants were harvested and tested for production of IL-4.

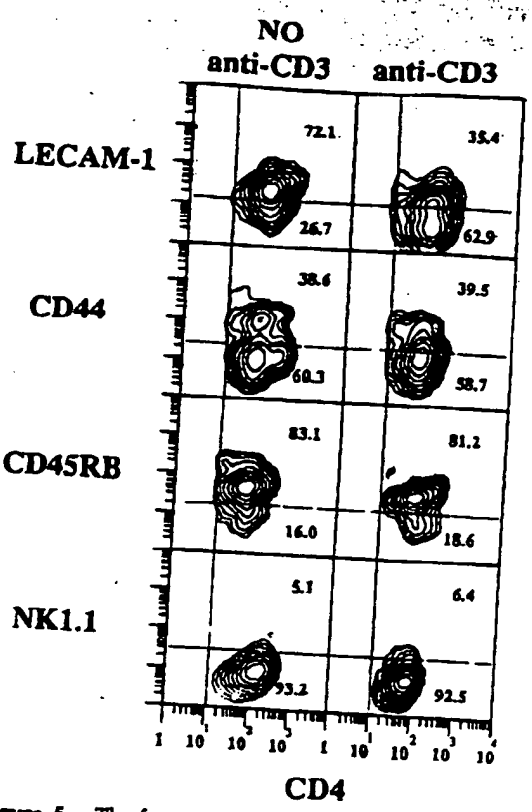


**Figure 5.** The frequency of $CD4^{pos}$,$LECAM\text{-}1^{dull}$ spleen cells is increased in response to anti-CD3 injection. CD4-enriched spleen T cells from uninjected mice or mice injected with anti-CD3 1.5 h earlier were stained with a mixture of PE anti-CD4 and FITC anti-LECAM-1, FITC anti-CD44, FITC CD45RB or biotinylated anti-NK1.1 and FITC-avidin. The percentages shown represent the proportion of bright and dull (LECAM-1, CD44, and CD45RB), or positive and negative (NK1.1) cells among the $CD4^{pos}$ T cells. The frequency of $NK1.1^{pos}$ cells in the experiment illustrated here was determined in the absence of a blocking anti-FcγRII/III antibody (2.4G2). However, in another experiment in which unlabeled 2.4G2 was added, there was no difference in the percent of $NK1.1^{pos}$ cells among $CD4^{pos}$ cells in uninjected and injected mice although the absolute percentages (4.5 and 4.8) were slightly lower than in the experiment shown here.

suggest that IL-4 production in response to injection with anti-CD3 is not mimicked by in vitro exposure to anti-CD3 even when the microenvironment of the interaction is preserved by the use of splenic fragments. One possibility that might account for the failure of in vitro anti-CD3 to promptly elicit IL-4 production in splenic fragments from uninjected mice is that the anti-CD3 might penetrate the fragment relatively slowly. To rule out this possibility, we prepared splenic fragments from mice that had been injected with anti-CD3 15 min before sacrifice. As noted above, anti-CD3 binds to T cells by this time in amounts as great as at 1.5 h after injection. Nonetheless, these fragments, even when cultured in anti-CD3, which should ensure a maintenance of anti-CD3 concentrations, fail to produce IL-4 in substantial amounts until 4 h of culture. These results further support the concept that the splenic fragment culture does not mimic the in vivo interaction of anti-CD3 with T cells that leads to prompt production of IL-4.

*Surface Phenotype of Cells That Produce IL-4 in Response to Injection of Anti-CD3.* We next wished to determine the phenotype of the spleen cells that produce IL-4 after in vivo treatment with anti-CD3. We first examined the composition of the spleen cells in uninjected mice and in mice injected with anti-CD3 1.5 h earlier. Fig. 5 shows that among the $CD4^{pos}$ T cells, there is a considerable increase in the frequency of $LECAM\text{-}1^{dull}$ cells in the spleens of anti-CD3–injected mice. No differences in the frequency of $CD44^{bright}$, $CD45RB^{dull}$, or $NK1.1^{pos}$ cells were noted, nor were there any obvious differences in the expression of these markers on $CD4^{neg}$ cells (data not shown).

Spleen cells from mice injected with anti-CD3 1.5 h earlier were separated into $CD4^{pos}$ and $CD8^{pos}$ populations and int a series of subpopulations of $CD4^{pos}$ cells. Using RT-PCR to analyze mRNA expression, IL-4–producing cells were found in the $CD4^{pos}$ but not the $CD8^{pos}$ group (Fig. 6 *A*). In a separate experiment, $CD4^{neg}$,$CD8^{neg}$ cells from anti-CD3–injected mice showed little or no IL-4 mRNA (data not shown). Among $CD4^{pos}$ cells, the IL-4 producers were in the group that were $LECAM\text{-}1^{dull}$, $CD44^{bright}$, and $CD45RB^{dull}$, consistent with the expression of an activated or memory phenotype (42). $CD8^{pos}$ T cells expressed more IFN-γ mRNA than $CD4^{pos}$ T cells. Nonetheless, among the $CD4^{pos}$ T cells, those that expressed IFN-γ mRNA had the same phenotype as those that expressed IL-4 mRNA. IL-2 mRNA was observed in both $CD4^{pos}$ and $CD8^{pos}$ T cells. Among the $CD4^{pos}$ T cells, the dominant phenotype of the IL-2 pr ducers was als $LECAM\text{-}1^{dull}$, $CD44^{bright}$ and $CD45RB^{dull}$.

In separate experiments, spleen cells from C57BL/6 donors injected 1.5 h earlier with anti-CD3 were s rted in $CD4^{pos}$, $NK1.1^{pos}$ and $CD4^{pos}$,$NK1.1^{neg}$ populations. The $CD4^{pos}$,

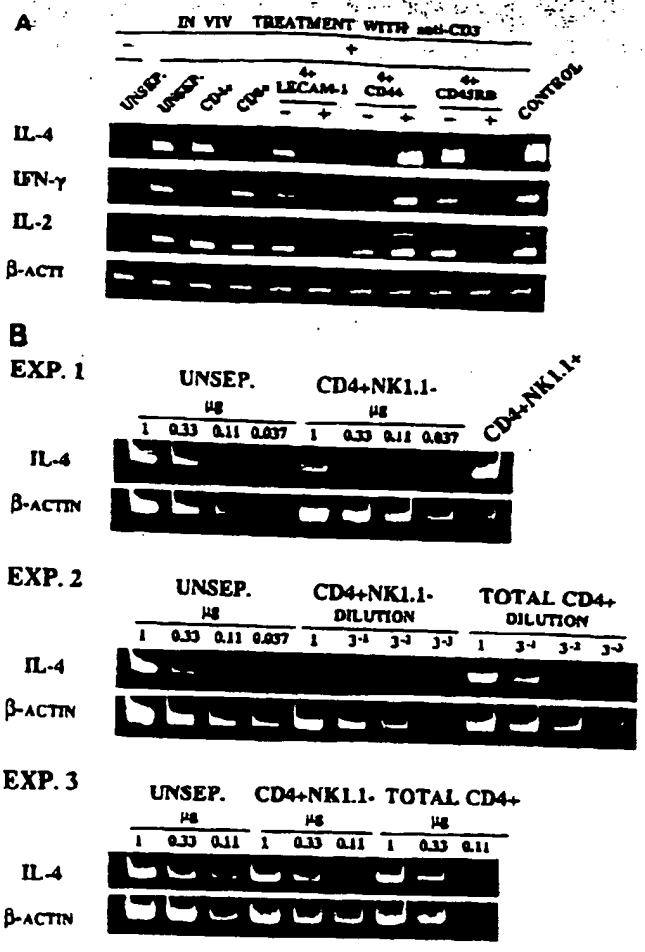


**Figure 6.** Expression of lymphokine mRNAs by T cell subsets from mice treated with anti-CD3. (*A*) Spleen cells from C57BL/6 mice injected with anti-CD3 1.5 h earlier were separated into $CD4^{pos}$ and $CD8^{pos}$ populations and into a series of subpopulations of $CD4^{pos}$ cells (LECAM-$1^{dull/bright}$, $CD44^{dull/bright}$ and $CD45RB^{dull/bright}$). During the sorting, spleen cells were kept at 4°C to insure stability of lymphokine mRNAs. Isolated mRNA was analyzed for expression of IL-4, IFN-γ, IL-2, and β-actin by using RT-PCR as described in Materials and Methods. As positive controls, mRNAs extracted from LT-1 cells and from purified T cells, stimulated with APC, anti-CD3 plus anti-IL-4 in vitro for 24 h were used for IL-4 and IFN-γ/IL-2, respectively. (*B*) Spleen cells from C57BL/6 mice injected with anti-CD3 1.5 h earlier were sorted into $CD4^{pos}$,$NK1.1^{pos}$ and $CD4^{pos}$,$NK1.1^{neg}$ populations (Exp. *1*). Spleen cells from C57BL/6 (Exp. *2*) or BALB/c (Exp. *3*) mice were sorted into total $CD4^{pos}$ and $CD4^{pos}$, $NK1.1^{neg}$ populations. A series of threefold diluted RNA samples were analyzed for expression of IL-4 and β-actin mRNA with RT-PCR. Where known, the amount of RNA samples used is reported in μg. UNSEP, total spleen cells before sorting.

$NK1.1^{pos}$ population, being relatively infrequent, yielded substantially less mRNA as judged by the RT-PCR amplification of actin mRNA. Despite this, IL-4 mRNA in this population was much greater than in the $CD4^{pos}$,$NK1.1^{neg}$ population (Fig. 6 *B*, exp. *1*). Although this result illustrates that $CD4^{pos}$,$NK1.1^{pos}$ T cells produce substantial amounts of IL-4 mRNA, they do not allow a direct determination of the total amounts of IL-4 mRNA in the $NK1.1^{pos}$ and $NK1.1^{neg}$ portions of the $CD4^{pos}$ cell population since the latter are much more numerous than the former. To test the magnitude of the contribution of $CD4^{pos}$,$NK1.1^{pos}$ cells to the production of IL-4 mRNA by the $CD4^{pos}$ cell population, we sorted C57BL/6 cells into total $CD4^{pos}$ and int $CD4^{pos}$,$NK1.1^{neg}$ subpopulations and compared the amounts of IL-4 mRNA by semiquantitative RT-PCR, using actin mRNA as a control. As seen in Fig. 6 *B* (exp. *2*), $CD4^{pos}$, $NK1.1^{neg}$ cells express approximately ninefold less IL-4 mRNA than do total $CD4^{pos}$ cells; this conclusion was verified by quantitative densitometric comparison of intensities IL-4 and β-actin RT-PCR products (data not shown). To control for specificity of staining with NK1.1, we separated BALB/c spleen cells into $CD4^{pos}$,$NK1.1^{neg}$ cells and total $CD4^{pos}$ cells. Since BALB/c cells do not express NK1.1, no difference in IL-4 mRNA between these populations would be expected. Indeed, no difference was observed (Fig. 6 *B*, exp. *3*). These results imply that the small population of $CD4^{pos}$,$NK1.1^{pos}$ cells produces the great majority of the IL-4 mRNA expressed by $CD4^{pos}$ cells from mice injected with anti-CD3. The data indicate that IL-4 production in the T cell population derives principally from $CD4^{pos}$,LECAM-$1^{dull}$, $CD44^{bright}$,$CD45RB^{dull}$,$NK1.1^{pos}$ cells.

*IL-4 Is Not Required for IL-4 Production in Response to Anti-CD3 Injection.* As noted in the beginning, IL-4 production in vitro requires or is markedly enhanced by the presence of IL-4 during priming (6, 7). Furthermore, anti-IL-4 treatment of mice at the time of immunization or infection diminishes subsequent IL-4 production upon rechallenge (9–11). To determine whether acute production of IL-4 in response to injection of anti-CD3 was similarly regulated, mice were treated with anti-IL-4 or with IL-4, IL-2, IFN-γ, or TGF-β beginning at 16 h before injection with anti-CD3. These mice showed no change in their expression of IL-4 mRNA in response to injection of anti-CD3 (Fig. 7 *A*). These results indicate that the factors that appear to regulate priming of T cells to become IL-4 producers do not control the acute production of IL-4 in response to injection of anti-CD3 antibody, thus suggesting that such cells could be a source of IL-4 for the regulation of priming of naive antigen-specific T cells.

Although production of IL-4 mRNA is not influenced by the lymphokines that normally regulate priming of IL-4-producing T cells, in vivo anti-CD3 stimulation does depend upon the interaction of T cells with cells expressing accessory ligands since CTLA-4Ig, which blocks the interaction of ligands for CD28 and CTLA-4 with these accessory receptors (35), substantially diminishes the IL-4 response to injection of anti-CD3 (Fig. 7 *B*).

*IL-4 Production Can Be Promptly Induced by Injection of SEB.* The capacity of anti-CD3 to rapidly induce IL-4, IFN-γ, and IL-2 production in spleen cells indicates that a potential source for the prompt production of these lymphokines exists that could be of importance in the process through which naive T cells develop into IL-4 or IFN-γ producers. However, anti-CD3 itself is not a physiologic stimulant and the frequency of T cells that could respond to conventional antigens is probably too low for the production of amounts of lymphokine sufficient to influence priming of naive T cells. We consid-

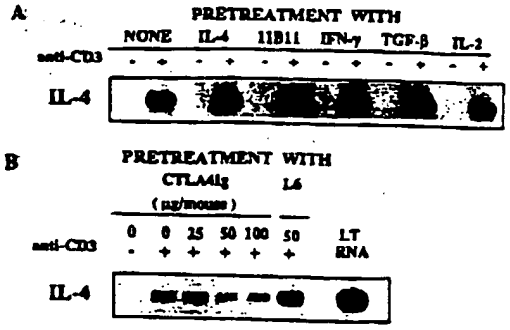


**Figure 7.** IL-4 is not required for IL-4 production in response to anti-CD3 injection but production is inhibited by CTLA-4Ig. (*A*) Mice were pretreated intraperitoneally with 9 mg of anti-IL-4 (11B4) or intravenously with $5 \times 10^6$ U of IL-4, 1 μg of IL-2, 1 μg of IFN-γ, or 2 μg of TGF-β 16 h and 1 h before injection of anti-CD3 or HBSS. (*B*) Mice were pretreated with CTLA-4Ig (0, 25, 50, 100 μg/mouse) or control human–mouse chimeric mAb, Chi-L6 (50 μg/mouse) intravenously 1 h before injection of anti-CD3. Spleens were removed at 1.5 h after injection of anti-CD3 for RNA extraction and IL-4 mRNA was measured by Northern blot analysis.

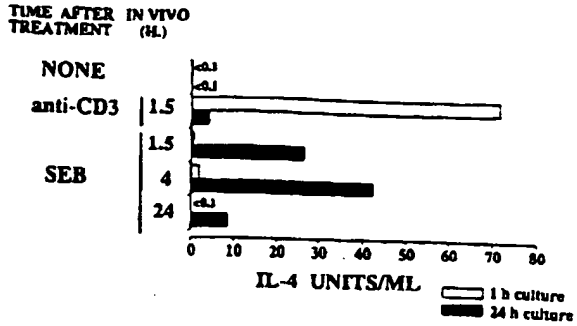


**Figure 9.** Production of IL-4 by T cells from SEB-injected mice. Five million spleen cells from mice injected with 1.33 μg of anti-CD3 (1.5 h earlier) or 100 μg of SEB (1.5, 4, 24 h earlier) were cultured in individual wells of 24-well plates for 1 h and 24 h without additional stimulus. Culture supernatants were harvested and tested for production of IL-4.

ered the possibility that superantigens might function like anti-CD3 since a response to these molecules could possibly make a substantial contribution to the lymphokine environment at the time of priming. Injection of SEB into C57BL/6 mice caused the appearance of IL-4 mRNA in the spleen within 1.5 h (Fig. 8 *A*). 200 μg of SEB caused a peak response; IL-4 mRNA could be detected by RT-PCR in response to 25-50 μg of SEB (Fig. 8 *B*). The amount of IL-4 mRNA and of IL-4 protein was less than in response to anti-CD3 and the time course of expression was somewhat prolonged, since mRNA and activity could still be detected in cells harvested 4 h after injection. However, by 24 h after injection, no IL-4 mRNA or activity was observed in spleen cells from mice injected with SEB or anti-CD3, suggesting that the overall response might be quite similar. Interestingly, T cells from SEB-injected mice required a more prolonged period of culture to produce IL-4 than did cells from anti-CD3–injected mice; more IL-4 was found in culture supernatants at 24 h of culture, without further stimulant, than after 1 h of culture (Fig. 9).

$CD4^{pos}$ T cells from mice injected with SEB were sorted into total $CD4^{pos}$ and $CD4^{pos}$,$NK1.1^{neg}$ subpopulations. IL-4 mRNA was substantially diminished in the $CD4^{pos}$, $NK1.1^{neg}$ cells in comparison with the total $CD4^{pos}$ subpopulation. Densitometric analysis of Southern-blotted PCR products indicated a threefold diminution, in comparison with total $CD4^{pos}$ cells in one experiment and a failure of $CD4^{pos}$, $NK1.1^{neg}$ to produce IL-4 in a second experiment (Fig. 10). These results indicate that the cells producing IL-4 in response to anti-CD3 and to SEB were of the same phenotype.

## Discussion

Primed $CD4^{pos}$ T cells, both in vitro and in vivo, generally exhibit either a Th1- or Th2-like phenotype (2, 43–48). Recently, the mechanisms by which a particular T cell lin-

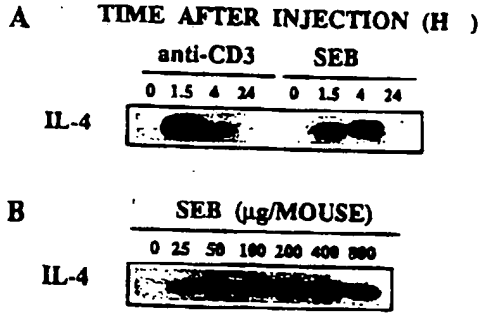


**Figure 8.** In vivo challenge with SEB can stimulate prompt production of IL-4. (*A*) C57BL/6 mice were challenged intravenously with 1.33 μg of anti-CD3 or 100 μg of SEB and spleens were removed for RNA extraction at indicated times. (*B*) C57BL/6 mice were injected with various doses of SEB as indicated and sacrificed at 4 h after injection to prepare total spleen RNA. IL-4 mRNA levels were analyzed by RT-PCR with Southern blot analysis.

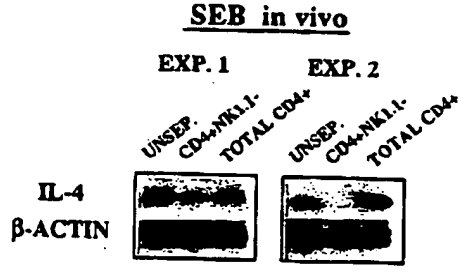


**Figure 10.** $CD4^{pos}$,$NK1.1^{pos}$ T cells promptly produce IL-4 in response to in vivo challenge with SEB. Spleen cells from C57BL/6 mice injected with 200 μg of SEB 4 h earlier were sorted into total $CD4^{pos}$ and $CD4^{pos}$, $NK1.1^{neg}$ populations. Expression of IL-4 and β-actin mRNA was measured by RT-PCR with Southern blot analysis. Two experiments are shown. In the first, 1 μg of RNA prepared from each group was used; in the second, 0.33 μg of RNA was used.

eage is steered down the path toward these distinctive phenotypes have been clarified both by studies of in vitro priming requirements (6–8) and by examining in vivo responses to infectious agents and to conventional antigens (9–12). These studies have emphasized the importance of cytokines themselves in the determination of a lymphokine-producing phenotype. IL-4 has been shown to be critical for priming cells to become IL-4 producers (6, 7) and IL-12 enhances priming for IFN-γ production (49, 50). In addition, IL-2, IFN-γ, and TGF-β have been shown to have effects on the priming process (4, 51–54). If we limit ourselves to the two lymphokines that have a dominant role, IL-4 and IL-12, a potential source of one of these, IL-12, available at the time of priming and produced under conditions that are appropriate for priming cells to become IFN-γ-producers, has been identified (49, 50). That is, macrophages infected with intracellular microorganisms or even exposed to heat-killed *Listeria monocytogenes* produce substantial amounts of IL-12 (49, 55). Since IFN-γ, produced by T cells primed in the presence of IL-12, enhances the capacity of macrophages to destroy intracellular pathogens, a logical link between production of the inducer and the effect of the product is established.

For IL-4, the situation is more complex. First, the inducer and the product are the same (6, 7). Second, IL-4 is known to be made mainly by activated T cells and by FcεRI$^{pos}$ cells, mainly cells with the morphology of basophils (17, 18). Neither of these cell populations appears ideally suited to be a physiologic source of IL-4 that could be used in the priming process to drive naive cells to become IL-4 producers. For the activated CD4$^{pos}$ T cells to do so would require that the inducing antigen could lead to their activation. Unless the inducing antigen had polyclonal stimulating capacities, IL-4 production in response to it would imply that the response was secondary, not primary, and would thus not solve the problem of how primary IL-4–dominated responses were induced. The difficulty about FcεRI$^{pos}$ cells being the principal source of IL-4 available at the time of priming is that the only established physiologic pathway through which these cells are stimulated to produce IL-4 is by cross-linkage of FcεRI or FcγRII/III (16, 17). In both cases, the cross-linkage would normally be dependent on antibodies of the IgE or IgG classes. Since the production of IgE (56), mouse IgG1 (57), and human IgG4 (58) is IL-4 dependent, an FcR-mediated stimulation of lymphokine production would appear to be likely only after the production of IL-4 had occurred. It is, of course, possible that non-FcR-mediated stimulation of basophils and/or mast cells stimulates IL-4 production; studies addressing this possibility are underway. Nonetheless, these arguments have led us to more seriously consider the possibility that cell types other than basophils and mast cells might be responsible for the initial burst of IL-4 production that plays a central role in the priming process.

We have confirmed the prior observations that spleen cells can produce large amounts of IL-4 mRNA in response to acute in vivo treatment with anti-CD3 (19, 20). Our studies reveal several quite interesting features of this response. First, in response to intravenous injection, IL-4 production appears to be mainly a property of spleen cells, although popliteal lymph nodes do show substantial IL-4 mRNA in response to f ot pad injection of anti-CD3. Even more striking is the fact that rapid induction of IL-4 mRNA and secretion of IL-4 in response to anti-CD3 is not mimicked by in vitro exposure f spleen cell suspensions or of spleen cell fragments to anti-CD3. Although this might still be explained by a critical microenvironmental requirement for stimulation of T cells to produce IL-4 promptly, it is equally consistent with the possibility that the cell that is responsible for lymphokine production is not in the spleen at the time of injection of anti-CD3 but only migrates there after stimulation. However, we have cultured blood cells in vitro with anti-CD3 and did not observe prompt production of IL-4 (data not shown) suggesting that homing of CD4$^{pos}$ peripheral blood T cells to the spleen after activation does not explain these results. For the moment, the difference between in vivo and in vitro responses to anti-CD3 are unexplained.

Our results implicate an unusual T cell population as the major producer of IL-4 in response to in vivo injection with anti-CD3. Thus, we observed that CD4$^{pos}$ T cells were the major cell population that expressed IL-4 mRNA in response to anti-CD3. Among the CD4$^{pos}$ cells, it was cells that expressed low levels of LECAM-1, high levels of CD44, and low levels of CD45RB that had virtually all the IL-4–producing capacity (Fig. 6 *A*). Although this phenotype is consistent with the IL-4–producing cell being a recently activated T cell (42), the observation that the great majority of IL-4 was made by the relatively small number of NK1.1$^{pos}$ cells in that population, suggests that prompt IL-4 pr duction in vivo may be mediated by a specialized cell population. Indeed, cells with this phenotype and related phenotypes have already been implicated as the major source of IL-4 among thymocytes (21, 59, 60). Thymic migrants within the spleen retain substantial IL-4–producing activity in response to in vitro treatment with anti-CD3, but only for a few days (60). It has been shown that a population of CD4$^{pos}$, NK1.1$^{pos}$ cells is present within the bone marrow (21). Although we found only modest amounts of IL-4 mRNA in bone marrow cells in response to injection of anti-CD3, it is possible that these cells only gain high level responsiveness in vivo upon redistribution to the periphery. However, we have not excluded the possibility that normal CD4$^{pos}$ T cells that become primed for IL-4 production in vivo acquire the expression of NK1.1, just as they become LECAM-1$^{dull}$, CD44$^{pos}$, and CD45RB$^{dull}$.

The alternative possibilities (i.e., a specialized cell population or a population of recently primed cells) are consistent with the observed lack of requirement of IL-4 at the time of stimulation for production of IL-4 in response to in vivo anti-CD3. Similarly, the finding that the production of IL-4 in response to anti-CD3 is inhibited by prior treatment of mice with CTLA-4Ig (Fig. 7 *B*) while indicating an important requirement for a costimulatory signal (34), does not distinguish between these two alternatives. It has been reported that the CD4$^{pos}$,NK1.1$^{pos}$ cell population displays a skewed expression of TCR-β chains, with Vβ8, Vβ7, and Vβ2

dominating (61). We are in the process of determining whether Vβ8 is also more highly represented among the IL-4 producing cells derived from in vivo challenge with anti-CD3.

The possibility that the $CD4^{pos}$,$NK1.1^{pos}$ cells described here make an important contribution to the production of the IL-4 that biases T cell responses to the Th2-like pathway would depend very greatly on the likelihood that such cells could be induced to produce IL-4 in response to natural immunization with agents that normally induce IL-4–dominated responses. We think it unlikely that responses to conventional T cell epitopes on these immunogens could recruit a sufficiently large fraction of the potential IL-4 producers to cause the secretion of enough IL-4 to affect a true primary response. However, the finding that SEB can induce a response rather like that mediated by anti-CD3, although of lower magnitude (Figs. 8 and 9), raises the possibility that immunogens that have associated superantigens might be able to stimulate a sufficient number of $CD4^{pos}$,$NK1.1^{pos}$ so that the local concentration f IL-4 available at the time of priming might be adequate for the differentiation of naive, antigen-specific T cells to develop into IL-4 producers. This would predict that agents such as nematodes and allergens would have associated superantigens.

Despite the many unresolved issues raised by these experiments, the demonstration of a cell population that promptly expresses IL-4 mRNA in vivo in response to receptor-ligation provides an important advance in the effort to determine the physiologic mechanisms through which immunization leads to $CD4^{pos}$ T cell responses dominated by Th1-like cells or Th2-like cells.

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